HPLC METHOD DEVELOPMENT FOR PHARMACEUTICALS

Edited by
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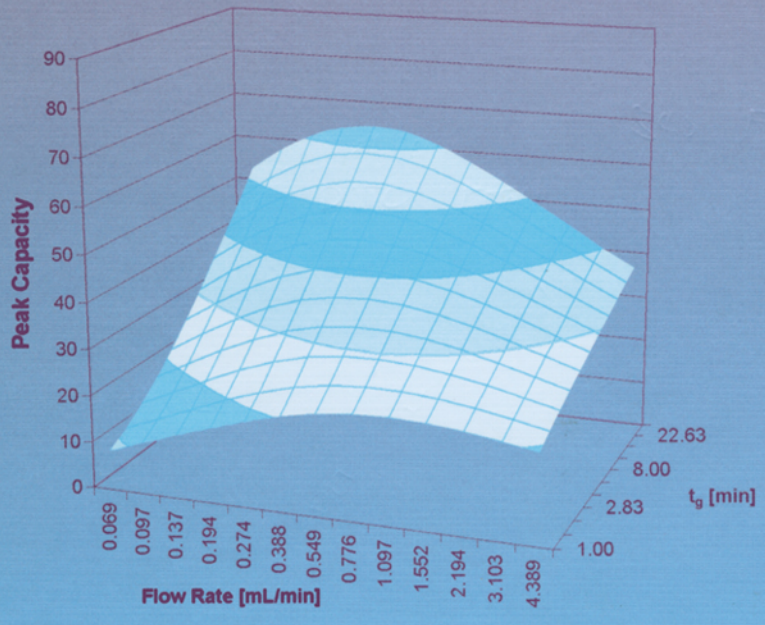

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HPLC METHOD DEVELOPMENT FOR PHARMACEUTICALS

This is Volume 8 of

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PREFACE

High pressure, or high performance, liquid chromatography (HPLC) is the method of choice for checking purity of new drug candidates, monitoring changes during scale up or revision of synthetic procedures, evaluating new formulations, and running control/assurance of the final drug product.

While numerous texts on HPLC are available, there are several unique concerns for pharmaceuticals that have not been discussed previously in one single book:

- Strategies have to be developed for instrument qualification and validation to meet various regulatory requirements.
- HPLC methods need to meet stringent validation requirements before they are utilized for any pharmaceutical evaluations.
- Impurities and degradation products to be separated are frequently not known and must be elucidated as part of the method development process.

The aim of this book is to provide an extensive overview of modern HPLC method development that addresses these unique concerns. For the purpose of providing a single source of information, an attempt has to been made to address all topics relevant to HPLC method development. The book is comprised of two major sections:

1. Review and update of the current state of the art and science of HPLC, including theory, modes of HPLC, column chemistry, retention mechanisms, chiral separations, modern instrumentation (including ultrahigh-pressure systems), and sample preparation.

Emphasis has been placed on implementation and on providing a practical perspective.

2. Focus on unique requirements for HPLC in a pharmaceutical setting. These include:
   - Strategies for software and hardware validation to allow for use in a regulated laboratory.
   - An overview of the pharmaceutical development process (clinical phases, chemical, and pharmaceutical development activities) to bring method development discussion into context.
   - A comprehensive discussion of how HPLC is used in each phase of pharmaceutical development and how methods are developed to support activities in each phase.
   - A discussion of method validation activities that provides an extension of ICH guidelines (i.e., provides an interpretation of ICH guidelines).
   - A comprehensive discussion of troubleshooting and prevention of problems.
   - A review of the emerging field of molecularly imprinted polymers and its potential applications in pharmaceutical analyses.

The book is intended to be particularly useful for both novice and experienced HPLC method development chemists in the pharmaceutical industry and for managers who are seeking to update their knowledge. We would like to thank the authors for their contributions that will make this book serve as a definitive reference source of HPLC method development for researchers, analysts, managers, and regulators of the pharmaceutical industry.

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1 OVERVIEW OF HPLC METHOD DEVELOPMENT FOR PHARMACEUTICALS

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I. INTRODUCTION

High-pressure, or high-performance, liquid chromatography (both are abbreviated as HPLC or simply LC; see references 1–2 as to proper usage of these terms) is predominantly used in the pharmaceutical industry for evaluations of a large variety of samples. It is the method of choice for checking purity of new chemical entities, monitoring changes

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in synthetic procedures or scale up, evaluating new formulations, and carrying out quality control/assurance of the final drug product.

The aim of this book is to provide an extensive overview of modern HPLC method development that incorporates these unique requirements. It is important to pay special attention to the following:

- Develop methods that would resolve unknown potential impurities and degradation products.
- Develop strategies for instrument qualification and validation to meet regulatory requirements.
- Provide stringent validation of HPLC methods before they are utilized routinely.

An attempt has been made to address various topics relevant to HPLC method development in a single text. While the focus of each chapter is on the current state of knowledge in HPLC, the emphasis is on implementation in a pharmaceutical setting.

II. THEORETICAL CONSIDERATIONS

Selectivity and detectability optimization have been the primary goals in HPLC separations for some time;[3,4] however, HPLC practitioners who are not trained in the physical sciences are reluctant to use theoretical considerations in method development. An attempt has been made in Chapter 2 to overcome this reluctance, using intuitive arguments. The essential fundamental theoretical concepts of HPLC are explained in a largely phenomenological way. The practical applicability of the concepts and equations are shown. Two main aspects comprise HPLC theory: kinetic aspect of chromatographic zone migration is responsible for band broadening, and thermodynamic aspect is responsible for the analyte retention in the column. The process of chromatographic zone dispersion in the column, usually called efficiency, is one of the important descriptors of chromatographic system. The rate of chromatographic zone migration is determined by the analyte competitive interactions with the stationary phase. In the isocratic separation mode this rate is constant, and the common analyte retention characteristic is the retention time (the ratio of the column length to the migration rate). Various equations have been provided to help the reader achieve optimal separations.

III. HPLC COLUMNS AND COLUMN PACKINGS

The surface chemistry of column packing as well as column design and performance are covered in Chapter 3. The section on column

chemistry deals with modern options of base materials as well as the commonly used approaches to evaluate the surface chemistry of a packing. Specific subsections are dedicated to the selectivity of reversed-phase packings, hydrophilic-interaction chromatography (HILIC), monolithic structures, and the reproducibility of modern packings. The reader can learn some basic principles on column selection in the section on speed and resolution. A brief discussion is also provided on very small diameter columns as well as preparative columns. Special attention has been given to items that are important for HPLC users in the pharmaceutical industry (also see Sections IV and V).

IV. COLUMN SELECTION

Reversed-phase (RP) LC methods are extensively utilized in the pharmaceutical industry. During HPLC method development, one of the biggest challenges is to select the stationary phase that has the desired selectivity, suitable repeatability, and stability (Chapter 4). Since more than 600 RP-LC column (brands or types) are available on the market, the selection of appropriate columns presents significant difficulties. Selectivity of the column and peak shapes of injected compounds is highly dependent on the characteristics of the column. Better understanding of the most significant features of the columns can facilitate fast solution of chromatographic problems that occur during method development (e.g., bad peak shape or poor resolution). Properties such as column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity, and the effect of metal impurities can be characterized by simple chromatographic tests. Calculating representative parameters of the tests allows classification of stationary phases with similar or dissimilar characteristics. The classification of stationary phases can be beneficial if a column has to be replaced with a similar selectivity column or with an orthogonal stationary phase. Such chromatographic test procedures can also be used to control the performance of a column at any time of its life cycle.

V. CHIRAL SEPARATIONS

The importance of chiral separation in pharmaceutical industry is well recognized and has been significantly emphasized by regulatory authorities. HPLC provides reliable and convenient methods of analyses for these compounds.[5,6] Separations of chiral active pharmaceutical ingredients (APIs) and related compounds by various methods are discussed in Chapter 5. Particular emphasis has been placed on the

separations of chiral compounds on chiral HPLC stationary phases (CSPs). Different types of chiral stationary phases, including polysaccharide derivatives, macrocyclic glycopeptides (antibiotics), cyclodextrins, Pirkle-type columns, proteins, ligand exchange, crown ethers, synthetic polymers/molecularly imprinted polymers (MIP), are discussed. As mentioned above, column selection is the most important and challenging step in method development. When choosing a column, the following should be considered: selectivity, mode of operation, compatibility of mobile phase with sample solvent, robustness, efficiency, loadability, and reproducibility. Separation mechanisms and method development for chiral molecules using these phases are also covered in this chapter.

Thanks to the rapid development of CSPs in the past few decades, the majority of chiral separations can be achieved on two types of universal columns, polysaccharide-based CSPs and macrocyclic glycopeptide CSPs. Both types of columns can be operated in reversed-phase, normal phase, and polar organic modes with complementary enantioselectivities. This makes it possible for a method developer who is new to the chiral separations field to build a practical level of expertise within a reasonably short period of time. A chiral database of various origins, as well as the column-screening approach, helps improve the efficiency for developing an effective chiral separation method. For difficult separation problems, a broader knowledge base and/or more experience are needed; however, it is still difficult to predict a separation of a new chiral molecule. This difficulty has been and will continue to be the driving force for development of new breakthrough technologies for chiral separations.

VI. CONTEMPORARY HPLC

Several basic components in a liquid chromatographic system are used for method development;[1,2] however, modern technology is quickly changing the landscape of method development by making improvements in "column chemistry," detection, and automation. The management of solvents and samples and the optimal use of the types of columns and detectors can have a significant impact on the time needed to develop a method and the quality and/or robustness of the method. Chapter 6 provides a brief description of a basic HPLC method development system, and then discusses in some detail modern ultra performance liquid chromatography (UPLC; it should be noted that better terminology of this technique would be ultrahigh-performance liquid chromatography) instrumentation components and how they are used to develop methods for complex samples encountered in today's pharmaceutical laboratory. Recent advances in this technology capitalize on sub-two micron particle column packings and automated approaches.

VII. HYPHENATED METHODS

The combination of HPLC with compound-specific detection by mass spectrometry (HPLC-MS or LC-MS; sometimes a slash is used instead of a hyphen: LC/MS) is discussed in Chapter 7; theory and application of modern LC-MS in its most commonly employed configurations are also covered. Included are discussions of the most widely applied ionization processes for LC-MS and the most useful and commonly used mass analyzers. Particular emphasis is given to method development for chromatography and the impact of employing LC-MS on the method development process. Also included is a discussion of gas-phase ion chemistry and how chromatography parameters can be employed to manipulate gas-phase ion chemistry to enhance LC-MS analysis. The relatively new combination of liquid chromatography with nuclear magnetic resonance spectroscopy (LC-NMR) is described in some detail. The discussion includes a critical examination of the costs and benefits of LC-NMR relative to conventional "tube" NMR with off-line sample isolation and purification. Finally, some helpful information is provided on the double hyphenated technique of LC-MS-NMR.

VIII. SAMPLE PREPARATION

Samples requiring HPLC analysis of pharmaceuticals can come in many forms: as neat chemical compounds such as APIs or drug substances, in a simple tablet or liquid matrix (the drug product) comprising a few solid or liquid excipients, complex drug delivery systems, and possibly pharmaceutical devices (see Chapter 8). In order to develop a rugged method, a statistically valid sampling plan should be developed.[2] The sample preparation method can be labor-intensive and require considerable planning to save time. Sample preparation is a critical step in the analysis of pharmaceutical products. In addition to developing methods to support these specific dosage forms, methods are required to support discovery and toxicological studies, requiring the isolation of pharmaceutical analytes from reaction by-products, animal feed, biological fluids and tissues, waste streams, and environmental samples. All of these require sample preparation prior to analysis. Sample preparation can be as simple as transferring the liquid analytes from the source container to an HPLC sample vial, or it can be quite complex requiring multiple steps such as solubilization extraction, concentration, cleanup, solvent transfer, and chemical modification. Regardless of the complexity, when the final sample is ready for injection into the HPLC it should be representative of the original sample, soluble in the mobile phase or solvents that are miscible with the mobile phase, solubilized in a solvent that provides sufficient stability for the duration of the analysis and any potential investigations,

free of particulates that could damage the HPLC system, at a concentration appropriate for the method of detection, and in a chemical form that is compatible with the mode of detection.

IX. INSTRUMENT QUALIFICATION AND SOFTWARE VALIDATION

Chapter 9 provides readers from different backgrounds (pharmaceutical scientists, quality assurance, and qualification personnel) an improved understanding of laws and regulations that apply to the pharmaceutical industry to ensure patient safety, and how they impact equipment qualification efforts. Different elements that cause competing interpretations of requirements and the confusion about exactly what is required to achieve compliance with regulations that pertain to equipment qualification are discussed. The discussion covers such widely used terms as calibration, qualification, and validation, while shedding light on what differences and similarities exist between equipment qualification, validating a method, and performing system suitability tests. A model is presented that can be used to ensure that qualification activities occur in a systematic organized manner and that also enables the reader to implement a scientifically sound decision-making process. An in-depth case study has been included that focuses on the critical aspects of different HPLC modules (solvent delivery system, detector, column compartment, and an injector/autosampler) from a qualification perspective.

X. PHARMACEUTICAL DEVELOPMENT

Basic method development has been covered in some earlier texts.[1–9] The evolutionary process of HPLC method development and utilization at each stage of the drug development process to fulfill the scientific, regulatory, and business needs of a pharmaceutical company is described in Chapter 10. There are three driving factors that determine the type of analytical techniques that are needed at each stage of the drug development process. These three factors are regulatory requirements, scientific requirements, and business requirements. The cutting edge of chemistry, biology, and physics is utilized to synthesize a new chemical entity, select an appropriate candidate for clinical studies, and develop new pharmaceutical formulations. Throughout the development process it is critical to learn quickly all the desired information about the compound under consideration. In this manner a therapeutic indication for the compound can be determined, as well as any potential adverse indications that may lead to the demise of the compound. The experiments that are conducted

during the discovery and the preclincial phases form the scientific foundation that provides the critical knowledge base for the compound. This "critical mass" of knowledge is utilized later in the development and clinical phases to ensure that a safe and effective product is developed. Worldwide, the pharmaceutical industry is one of the most heavily regulated industries. No prescription product can be marketed or sold without the permission of the relevant regulatory authority. As a result, one of the key drivers in bringing a pharmaceutical substance to market is complying with all of the relevant regulatory requirements. These requirements generally call for the generation of an information package, based on a series of clinical and analytical studies that demonstrate the safety and efficacy of the product. In addition, the pharmaceutical organization must demonstrate that the required quality systems that allow for the development and continued production of such products are in place. These quality systems tend to slow the development process and cost additional resources, but the cost of not implementing the required quality system can be much greater. The end result of any pharmaceutical venture is the generation of profit. In order to realize such a profit, the product must be produced, tested, and released as inexpensively as possible. This drives the selection of synthetic processes and analytical testing methodologies utilized for the final product. The phrase "time is money" can be applied here. Since there is a finite lifetime for a patented product on the market, this requires fast development of processes and analytical methodologies.

XI. DRUG DISCOVERY

In the drug discovery phase, the goal is to learn as much as possible about many compounds of interest in a short time. Among many criteria, obtaining experimental pharmacokinetics (PK) data from laboratory animals early in the discovery is critical to the evaluation of a drug candidate. Thus, a fast turnaround time from sample receipt to the PK report is crucial (see Chapter 11). Developing an accurate and fast analytical method for measuring the concentrations of a compound in plasma or tissue is the first step in determination of the PK of a compound. This chapter provides practical details on rapidly developing a reliable bioanalytical method by combining HPLC with tandem mass spectrometry (LC-MS/MS) to support in vivo PK screens and studies. Some detailed discussion is provided in terms of selection of the HPLC mobile phase, column, and sample preparation strategy. Some common issues in terms of matrix effect and background interference are also presented, and possible solutions are given. Four levels of assay criteria have been offered to meet the needs at various stages in new drug discovery.

XII. EARLY PHASE METHOD DEVELOPMENT

The primary goal of early phase method development is to gain a fundamental knowledge of the chemistry of drug substances and drug products to facilitate optimization of synthetic schemes and drug product formulations. At the same time, methods are required for release and stability studies to support clinical trials and must assure that products are safe in vivo. Ultimately, the knowledge gained during early development translates into designing control methods for commercial supplies that assure patient safety and efficacy. Chapter 12 describes an iterative and systematic approach to HPLC method development, designed to meet the specified goals. HPLC methods are necessary to support clinical release and stability activities and evolutionary synthesis and formulation optimization. To address changes in the impurities and degradation product profiles generated during these activities, a systematic approach to method development using an array of methods is advocated as a means of obtaining full knowledge of drug substance and drug product chemistry. The approach places heavy emphasis on the use of orthogonal screening as a central activity, to gather the knowledge required as a prerequisite to developing methods suitable for the control of commercial supplies of drug substance and drug product. Key elements of the approach include the generation of degradation products via forced decomposition and a continual evaluation of samples generated during the early development cycle.

XIII. LATE PHASE DEVELOPMENT

The late-phase methods are filed with regulatory authorities and are used for stability studies and for the release of the drug product (DP) or drug substance (DS) validation batches (see Chapter 13). For release testing of production batches, the methods are generally transferred to the operational quality control (QC) laboratories. In the ideal case, the analytical methods should last for the entire product lifetime. Therefore, the aim of late-phase method development is to develop fast, robust, reliable, and transferable HPLC methods. In this context, it is crucial to devote adequate time, thoughts, and resources to the development of late-phase analytical methods. In most cases, the development group tries to complete its task as well as possible with the talent and time available to them. The method is validated according to standard operating procedures and transferred to the application labs. The transfer exercise is considered to be the real challenge of the method. Both the development and the application labs are usually very eager to know the outcome of the transfer activity. However, many method-related surprises are frequently observed at this stage and have to be resolved, if possible. In some cases with severe issues, the method transfer fails and the method

has to be redeveloped. The resulting delay may heavily impact the product development timelines and even jeopardize the filing date. To avoid these delays, good communication between the method development and method utilization groups is necessary.

XIV. IN-PROCESS TESTING

Various types of in-process controls (IPCs) are used throughout the pharmaceutical industry to ensure that intermediate steps are complete prior to the end of a process (see Chapter 14). The main goal of conducting an IPC is to ensure that the material produced at the end of the process is acceptable from the standpoint of quality and yield. During the manufacture of drug substances (either in research, development, or manufacturing) this can involve utilization of a number of analytical techniques, depending on which stage the process is being monitored. A typical chemical process may consist of any of the following steps: reaction, separation, crystallization, and isolation/drying. Each step may entail an in-process test to ensure that it has been completed to a level that will allow the process to continue successfully. Alternatively, the IPC will alert that the process has not been completed successfully and that further action is required prior to proceeding to the next phase. In cases where IPCs show that a given step does not meet specifications, the corrective action has to be taken. In production, the regulations are more strict and material that does not meet specifications has to be reprocessed by repeating a prior step that is part of the process or reworked by processing the material through a known filed procedure.

XV. METHOD DEVELOPMENT FOR BIOMOLECULES

Various modes of liquid chromatography, including RPLC, HIC, ion-exchange chromatography (IEC), and size exclusion chromatography (SEC) are often applied to the separation of large biomolecules.[10] Sample complexity not only makes these separations difficult, but makes LC method development an arduous task. At best, the bioanalyst must first use knowledge of the sample to decide what needs to be separated; then a separation mode can be selected. Once the separation mode is chosen, specific method development steps are often little more than trial-and-error. While general LC method development guidelines are a useful starting place, additional characteristics that must be considered are presented. Discussed here are the varying nature of biomolecule sample properties and the role of solute hydrophobicity in achieving adequate resolution in each of the common modes of LC. Emphasis has been placed on analytical separation of peptides and proteins.

XVI. METHOD VALIDATION

Validation is the process that helps establish, by laboratory studies, that the performance characteristics of a method meet the requirements for the intended application. It provides documented evidence that the method performs for the intended purpose (see Chapter 16). The validation characteristics and the acceptance criteria to be applied in validation of HPLC methods for New Drug Application filing and marketed products should comply with the international guidelines on method validation. The choice of characteristics to be evaluated depends on the purpose of the analytical method and the product development stage that the analytical method is supporting. It is recognized that methods are often evolutionary and that methods in early development will generally require less extensive validation than methods in final development. To assure smooth performance, it is important that the analytical method clearly document the steps involved in performance of analysis. Validated analytical methods should be used for the analysis of drug substance and drug product. Appropriate validation of analytical methods for other materials (e.g., intermediates, excipients, preservatives, etc.) used in the production of drug substances and drug products should also be considered.

XVII. TROUBLESHOOTING

An overview of HPLC troubleshooting and a summary of guidelines for system maintenance are given in Chapter 17. Troubleshooting problems can be classified into four major categories: problems observed in a chromatogram, operating parameters, leaks, and pressure problems. The chapter concludes with a section on how to avoid problems by routine and regular maintenance. Routine maintenance procedures that can be performed by the user are emphasized. After describing each problem, symptoms and possible solutions are proposed. The reader is also referred to textbooks and magazine columns. Discussion is focused primarily on RP columns since they are used predominantly in the pharmaceutical industry today. The readers are encouraged to find the relevant problem area in the outline and go to that section and read about possible causes and solutions.

XVIII. MOLECULARLY IMPRINTED POLYMERS

Molecularly imprinted polymers (MIPs) are materials that mimic biological receptors with synthetic recognition sites that exhibit predetermined selectivity toward analyte(s) of interest. MIPs have become increasingly popular in recent years and have been applied as selective sorbents for

extractions, chromatography, and in other areas where high specificity is required. MIPs have been implemented in various applications, including sample preparation, as stationary phases for analytical separations and analyte recognition materials in affinity assays. Chapter 18 presents a summary of factors of importance in the synthesis and use of MIPs, describes various synthetic approaches, and reviews recent advances in the field of molecularly imprinted materials and pharmaceutical applications.

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2 HPLC THEORY

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ABSTRACT

The behavior of the analyte in HPLC columns is discussed from the point of view of analyte distribution between the adsorbent and the bulk liquid phase. Different retention mechanisms are considered and expressions describing the analyte retention volume are derived for each selected retention mechanism. Methodology of the incorporation of multi-equilibrium processes in the analyte retention expression is suggested for linear chromatography conditions. The description of eluent components' adsorption is discussed on the basis of the excess adsorption process. The applicability of the excess adsorption description without introduction of the adsorbed layer model or any other convention to the chromatographic process is shown.

I. INTRODUCTION

The most essential fundamental theoretical concepts of HPLC will be explained in this chapter in a mainly phenomenological way. The

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practical applicability of those concepts and equations will be shown. There is an understandable reluctance in making use of theory among some people not trained in the physical sciences. A mere realization of the importance is of little value when faced with a set of partial derivative equations. We will try to conquer this reluctance by using intuitive arguments.

Two main aspects comprise HPLC theory: kinetic aspect of chromatographic zone migration is responsible for the band broadening and thermodynamic aspects are responsible for the analyte retention in the column. The process of chromatographic zone dispersion in the column, usually called efficiency, is one of the important descriptors of a chromatographic system.

The velocity of the chromatographic zone migration is determined by the analyte's competitive interactions with the stationary phase surface. In the isocratic separation mode this velocity is constant and the common analyte retention characteristic is the retention time (the ratio of the column length to the migration velocity).

A simple approach to the separation from the point of view of the analytical chemist is generally the achievement of the complete separation or in other words the improvement of the resolution.

II. BASIC CHROMATOGRAPHIC DESCRIPTORS

Four major descriptors are commonly used to report characteristics of the chromatographic column, system, and particular separation:

1. Retention factor (k')
2. Efficiency (N)
3. Selectivity (α)
4. Resolution (R)

Retention factor (k') is the unitless measure of the retention of a particular compound in a particular chromatographic system at given conditions defined as

$$k' = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0} \tag{1}$$

where V_R is the analyte retention volume, V_0 the volume of the liquid phase in the chromatographic system, t_R the analyte retention time, and t_0 sometimes defined as the retention time of non-retained analytes. Retention factor is convenient since it is independent of the column dimensions and mobile phase flow rate. Note that all other chromatographic conditions significantly affect analyte retention.

Efficiency is the measure of the degree of peak dispersion in a particular column, as such it is essentially the characteristic of the column. Efficiency is expressed as the number of theoretical plates (N) calculated as

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{2}$$

where t_R is the analyte retention time and w the peak width at the baseline.

Selectivity (α) is the ability of chromatographic system to discriminate two different analytes. It is defined as the ratio of corresponding capacity factors

$$\alpha = \frac{k'_2}{k'_1} \tag{3}$$

Resolution (R) is a combined measure of the separation of two compounds and includes peak dispersion and some form of selectivity. Resolution is defined as

$$R = 2\frac{t_2 - t_1}{w_2 + w_1} \tag{4}$$

So far chromatographic descriptors were only introduced. In the following sections we discuss their functions, specifics, and relationships with different chromatographic and thermodynamic parameters.

III. EFFICIENCY

The most rigorous discussion of the formation of a chromatographic zone and the mathematical description of zone broadening is given in reference 1. Here we only discuss practically important and useful equations.

If all specific processes, such as surface interactions, analyte ionization, etc., have fast rate constants and thermodynamic equilibrium, molecular diffusion is the only parameter of kinetic concern. If column properties could be considered isotropic, then we expect symmetrical peaks of a Gaussian shape (Figure 1), and the variance of this peak is proportional to the diffusion coefficient:

$$\sigma^2 = 2Dt \tag{5}$$

At given linear velocity (v) the component moves through the column with length (L) during the time (t), or

$$L = vt \tag{6}$$

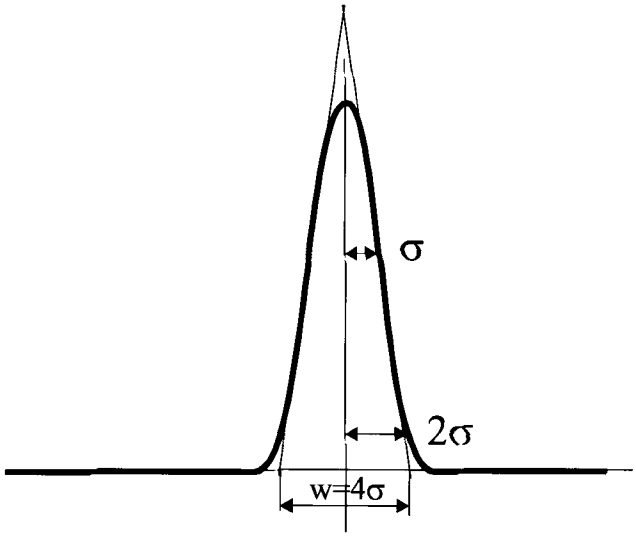

FIGURE 1 Chromatographic peak with symmetrical Gaussian band broadening.

Substituting t from Equation (6) in Equation (5) we get

$$\sigma^2 = \left(\frac{2D}{v}\right)L \tag{7}$$

The expression $2D/v$ has units of length and is essentially the measure of band spreading at given velocity at the distance L of the column. This parameter is essentially the *height equivalent to the theoretical plate* and could be denoted as H, i.e.,

$$H = \frac{\sigma^2}{L} \tag{8}$$

In the column there are several different processes leading to the band spreading phenomena, such as multipath effect, molecular diffusion, displacement in the porous beds, secondary equilibria, and others. Each of these processes introduces its own degree of variance in the overall band spreading process. Usually these processes are assumed to be independent, and based on the fundamental statistical law, overall band spreading (variance) is equal to the sum of the variances for each independent process:

$$\sigma^2_{\text{tot}} = \sum \sigma_i^2 \tag{9}$$

Further we will discuss the total variance in all cases.

In the form of Equation (9) the definition of H is exactly identical to the plate height as it evolved from the distillation theory and was

brought to chromatography by Martin and Synge.[2] If H is the theoretical plate height we can determine the total number of the theoretical plates in the column as

$$N = \frac{L}{H} \Rightarrow N = \left(\frac{L}{\sigma}\right)^2 \tag{10}$$

In linear chromatography each analyte travels through the column with constant velocity (u_c). Using this velocity we can express the analyte retention time as

$$t_R = \frac{L}{u_c} \tag{11}$$

Similarly, we can define the time necessary to move the analyte zone through the column a distance of one σ (Figure 1) as τ.

$$\tau = \frac{\sigma}{u_c} \tag{12}$$

Substituting both Equations (11) and (12) into Equation (10) we get

$$N = \left(\frac{t_R}{\tau}\right)^2 \tag{13}$$

Parameter τ in Equation (13) is the fraction of peak width equivalent to the standard deviation and is expressed in the same units as retention time. Since we considered symmetrical band broadening of a Gaussian shape we can use the Gaussian function to relate its standard deviation (i.e., the peak has half of the peak width at 0.609 of its height) to more easily measurable quantities. The most commonly used points are the so called peak width at the baseline, which is actually the distance between the points of intersections of the tangents to the peaks inflection points with the baseline (shown in Figure 1). This distance is equal to the four standard deviations and the final equation for the efficiency will be

$$N = 16\left(\frac{t_r}{w_b}\right)^2 \tag{14}$$

Another convenient point is the peak width at the half height. From the same Gaussian function the peak width at half height is 2.355 times longer than the standard deviation of the same peak and the resulting formula for the number of the theoretical plates will be

$$N = 5.545\left(\frac{t_R}{w_{(1/2)h}}\right)^2 \tag{15}$$

Efficiency is mainly a column-specific parameter. In gas chromatography column efficiency is highly dependent on the mobile phase flow rate. In HPLC due to much higher viscosity, the variations of the flow rate does not affect column efficiency significantly within the applicable flow rate region.

The geometry of the packing material and uniformity and density of the column packing are the main factors defining the efficiency of a particular column.

There is no clear fundamental relationship between the particle diameter and the expected column efficiency, but phenomenologically we can expect an increase of the efficiency with a decrease of the particle diameter, since there is a decrease of the gap between the average pore size in the packing material and the effective size of interparticle pores, which leads to a more uniform flow inside and around the particles.

From Figure 2 it is obvious that the smaller the particles the lower the theoretical plate height and the higher the efficiency. The general form of the shown dependence is known as the Van Deemter function (Equation (16)), which has the following mathematical form

$$H = \mathrm{A} + \frac{\mathrm{B}}{v} + \mathrm{C}v \tag{16}$$

where v is the linear flow velocity, and A, B, and C are constants for given column and mobile phase.

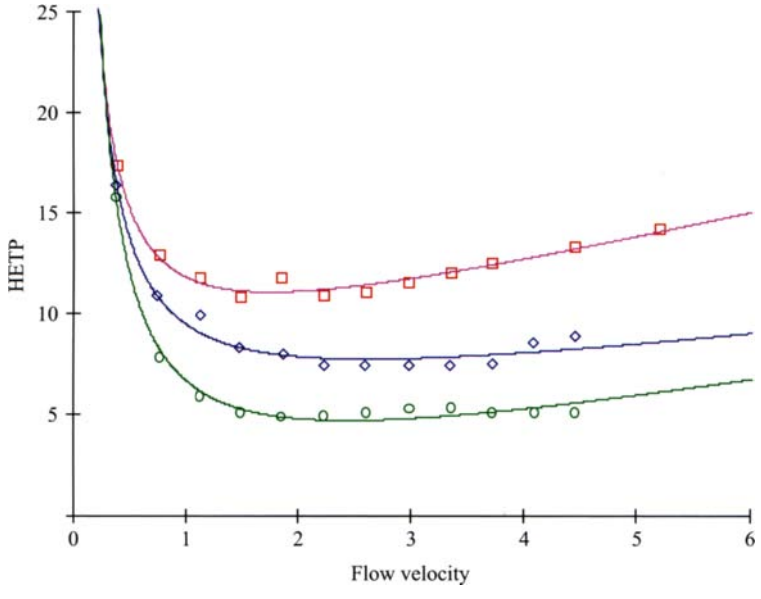

FIGURE 2 **The dependence of the theoretical plate height on the flow velocity for columns packed with particles of different diameter. (5μ – squares; 3μ – rhombs; 1.8μ – circles). (Reprinted with permission from reference 20.)**

Three terms of the above equation essentially represent three different processes that contribute to the overall chromatographic band broadening.

A. Multipath effect or eddy diffusion
B. Molecular diffusion
C. Mass-transfer

The multipath effect is a flow independent term, which defines the ability of different molecules to travel through the porous media with paths of different length.

The molecular diffusion term is inversely proportional to the flow rate, which means that the slower the flow rate the longer the component stays in the column and the more time the molecular diffusion process has to broaden the peak.

The mass-transfer term is proportional to the flow rate, which means that the faster the flow the higher the band broadening due to a bigger lag between a retained molecule compared to a molecule moving with the flow. Schematically superposition of all three processes is shown in Figure 3.

As can be seen from the comparison of Figures 2 and 3, all dependencies of the column efficiency on the flow rate follow a theoretical Van Deemter curve. In theory there is an optimum flow rate that corresponds to the highest efficiency (the lowest theoretical plate height).

As one can see from Figure 2, the lower the particle diameter the wider the range of flow rates where the highest column efficiency is achieved. Column packed with smaller particles can work with high efficiency at faster flow rates, because the mass-transfer term is low for these columns. Essentially this means that retention equilibrium is achieved much faster in these columns.

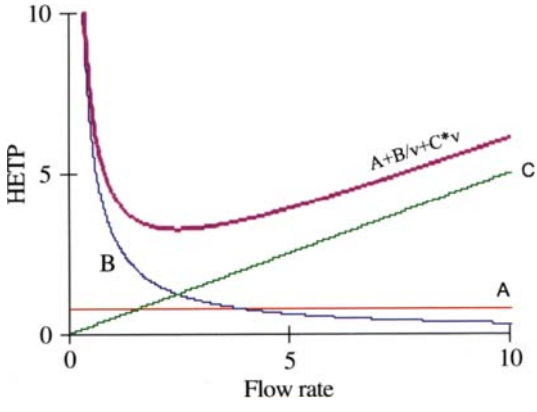

FIGURE 3 Schematic of the Van Deemter function and its components.

Faster flow rates mean higher flow resistance and higher backpressure. It is a modern trend to work with the smaller particles at high linear velocity. The overall efficiency of the columns packed with smaller particles is not much higher. If we compare a conventional 15 cm column with 4.6 mm internal diameter packed with 5μ particles with new a column of 15 cm length and 2 mm internal diameter packed with 1.7μ particles we observe that the average efficiency of the first column is between 12,000 and 15,000 theoretical plates, for the second column the efficiency is not much higher, it is on the level of 15,000–18,000 theoretical plates. This slight increase of the efficiency can hardly improve any separation, but if we compare the run times at the same volumetric flow rate on both columns we will see that the separation on the second column is achieved more than five times faster.

Of course, the ability to increase u depends on the pressure capabilities of the instrument, since pressure is directly proportional to velocity:

$$\Delta P = \frac{uL\eta\phi}{d_p^2} \tag{17}$$

where ΔP is the pressure drop across the column, η the viscosity, and ϕ the flow resistance factor. Therefore, the fastest possible separation requires that the maximum pressure allowed by the instrument be used, assuming the resolution requirement can be met. This also means the speed of analysis is limited by that maximum pressure. As a result, one wants to make the most of the pressure available to him by reducing the pressure drop across the column as much as possible.

To limit analysis time the shortest possible column should be used. There is another reason to limit column length – the pressure drop. Shorter columns have lower pressure requirements, allowing the use of the pressure to gain an advantage in speed. It must be kept in mind, though, that N will decrease as u increases, meaning that at faster velocities longer columns are necessary to give the required theoretical plates, thus requiring greater pressures.

IV. RESOLUTION

In the introduction section we defined the term resolution as the ability of the column to resolve two analytes into separate peaks (or chromatographic zones). In more general form than it was given before, the resolution can be defined as half of the distance between the centers of gravity of two chromatographic zones related to the sum of their standard deviations

$$R = \frac{X_2 - X_1}{2(\sigma_1 + \sigma_2)} \tag{18}$$

In case of symmetrical peaks we can substitute centers of peak gravity with the peak maxima and using the relationship between Gaussian peak width and its standard deviation we get a common expression for the resolution:

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_2 + w_1)} \tag{19}$$

If we use expression (14) for substitution of the peak width in Equation (19) we will get as a result

$$R = \frac{t_{R2} - t_{R1}}{t_{R2} + t_{R1}} \frac{\sqrt{N}}{2} \tag{20}$$

Expression (20) demonstrates that resolution is proportional to the square root of the efficiency.

From the practical point of view, in case of the lack of resolution in some specific separation there are generally two ways to improve it: increase the efficiency, or increase the selectivity. The efficiency is proportional to the column length, the longer the column the higher the efficiency, but as Equation (20) shows the increase of the efficiency increases the resolution only as a square root function (as illustrated in Figure 4). At the same time the increase in the column length leads to an increase of the flow resistance and backpressure, which limits the total possible column length.

If we assume that the peak widths of two adjacent peaks are approximately equal, we can rewrite expression (18) in the form:

$$R = \frac{X_2 - X_1}{4\sigma} \tag{21}$$

For symmetrical chromatographic bands this is the ratio of the distance between peaks maxima to the peak width. The distance between peak

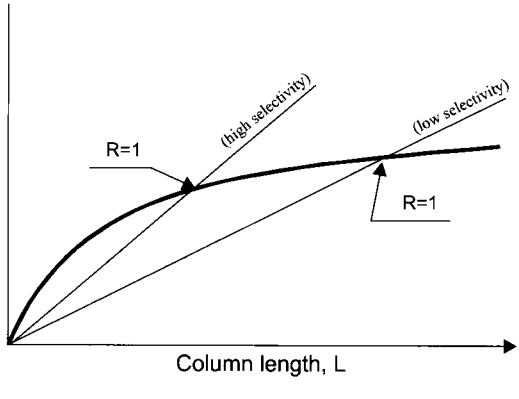

FIGURE 4 Relationship between the resolution, selectivity, and the column length.

maxima is proportional to the distance of the chromatographic zone migration and the peak width is proportional to the square root of this distance. Figure 4 illustrates this relationship.

At low selectivity, to achieve the same resolution, we have to use a longer column to compensate selectivity with higher efficiency and apply higher pressure. The relationship between the column length, mobile phase viscosity, and the backpressure is given by the following relationship, which is a variation of the Kozeny–Carman equation:

$$\Delta p = \frac{\varphi \eta \langle v \rangle L}{d_p^2} \tag{22}$$

where η is the viscosity of the mobile phase, L the column length, d_p the particle diameter of the packing material, $\langle v \rangle$ the mobile phase average flow velocity, and φ an empirical coefficient dependent on the column porosity (usually between 500 and 1000). Expression (17) predicts a linear increase of the backpressure with an increase of the flow rate, column length, and mobile phase viscosity. The decrease of the particle diameter, on the other hand, leads to a quadratic increase of the column backpressure.

V. MAIN COMPONENTS OF THE RETENTION MECHANISM

In the introductory section, the main chromatographic descriptors generally used in routine HPLC work were briefly discussed. Retention factor and selectivity are the parameters related to the analyte interaction with the stationary phase and reflect the thermodynamic properties of a chromatographic system. Retention factor is calculated using expression (1) from the analyte retention time or retention volume and the total volume of the liquid in the column. Retention time is essentially equivalent to the retention volume provided that the mobile phase flow rate is constant throughout the whole separation process.

Part of the total retention volume of the analyte is the void volume. Even if the analyte molecules do not interact with the column packing material the analyte needs some time to pass through the column. This time is usually called hold-up time, or dead time, or void time. The corresponding volume is the void volume, or the volume of the liquid phase in the column, or dead volume. Analyte retention volume, which exceeds the column void volume, is essentially the volume of the mobile phase which had passed through the column while analyte molecules were retained by the packing material.

To derive the relationship of the analyte retention with the thermodynamic properties of the chromatographic system the mechanism of the analyte behavior in the column should be determined. The

mechanism and the theoretical description of the analyte retention in HPLC has been the subject of many publications and different research groups and scientific schools are still in disagreement on what is the most realistic retention mechanism and what is the best theory to describe the analyte retention and if possible predict its behavior.[3,4] Almost 30 years ago, Colin and Guiochon mentioned in a review[5] that there are essentially three possible ways to model separation mechanism. The first is analyte partitioning between mobile and stationary phases, the second is the adsorption of the analyte on the surface of non-polar adsorbent, and the third, suggested by Knox and Pryde,[6] is based on the preferential adsorption of the organic mobile phase modifier on the adsorbent surface followed by the analyte partitioning in this adsorbed layer.

Partitioning is the first and probably the simplest model of the retention mechanism. It assumes the existence of two different phases (mobile and stationary) and instant equilibrium of the analyte partitioning between these phases. The simple phenomenological interpretation of the dynamic partitioning process was also introduced at about the same time. Probably the most consistent and understandable description of this theory is given by C. Cramers, A. Keulemans, and H. McNair in 1961 in their chapter "Techniques of Gas Chromatography."[7] The analyte partition coefficient is defined as

$$K = \frac{c_S}{c_M} \tag{23}$$

And the analyte capacity factor is defined as the ratio of the total amount of analyte in the stationary phase to the total amount of analyte in the mobile phase

$$k' = \frac{q_S}{q_M} = \frac{V_S c_S}{V_M c_M} = \frac{V_S}{V_M} K \tag{24}$$

The fraction of the analyte in the mobile phase can be written in the form

$$R_f = \frac{q_M}{q_S + q_M} = \frac{1}{1 + k'} \tag{25}$$

and regarded as a retardation factor. If it is assumed that R_f could be considered as the fraction of time that the component spends in the mobile phase, and multiplying the mobile phase velocity by R_f, the average component velocity in the column is obtained:

$$u_C = u \frac{1}{1 + k'} \tag{26}$$

where u is the mobile phase linear velocity and u_c the velocity of the analyte. The retention time is the ratio of the column length and analyte velocity, thus

$$t_R = \frac{L}{u}(1+k') = t_0(1+k') \tag{27}$$

Converting expression (27) into retention volumes using $V_R=Ft_R$ and $V_M=Ft_0$, where F is the mobile phase flow rate, and combining with Equation (24) we get

$$V_R = V_M + V_S K \tag{28}$$

These mnemonic derivations appear in all textbooks dealing with gas and liquid chromatography. Equation (28) describes the retention of the analyte, which undergoes only one process of ideal partitioning between well-defined mobile and stationary phases.

In gas chromatography the analyte partitioning between mobile gas phase and stationary liquid phase is a real retention mechanism, and phase parameters, such as volume, thickness, internal diameter, etc., are known. In liquid chromatography, on the other hand, just the question of simply correct definition of the mobile phase volume has been a subject of continuous debate in the last 40 years.[8–11] The assumption that the retardation factor, R_f, which is a quantitative ratio, could be considered as the fraction of time that the component spends in the mobile phase is not obvious either.

The phenomenological description of the retention mechanism discussed above is only applicable for a system with a single partitioning process and well-defined stationary and mobile phases. A more general method for the derivation of the retention function is based on the solution of column mass balance.

VI. GENERAL COLUMN MASS BALANCE

Analyte transport through the HPLC column is considered to be one-dimensional along the axis x of the column, as shown in Figure 5. The analyte in the column is considered to be in instantaneous thermodynamic equilibrium. Various processes in which analyte could be involved (e.g., ionization, solvation, etc.) are also considered to be at the equilibrium. To simplify the discussion we assume the absence of axial analyte dispersion. This, in some cases, allows for the analytical solution of the mass balance equation.

The following assumptions are made:

1. Molar volumes of the analyte and mobile phase components are constant and the compressibility of the liquid phase is negligible.

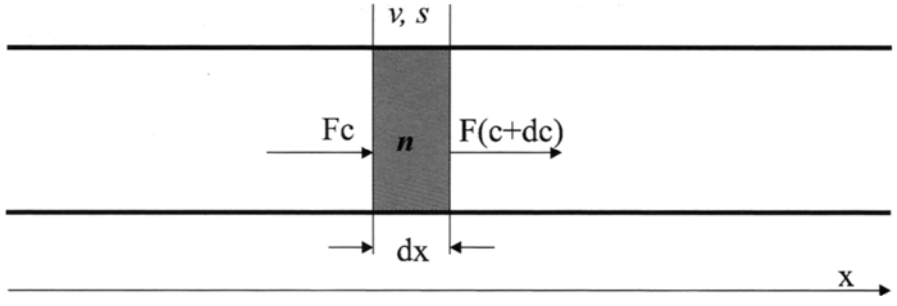

FIGURE 5 **Illustration of the column slice for construction of mass balance. Mobile phase flow *F* in mL/min; analyte concentration *c* in mol/L; *n* is the analyte accumulation in the slice *dx* in mole; *v* is the mobile phase volume in the slice *dx* expressed as V_0/L, where *L* is the column length; s is the adsorbent surface area in the slice *dx*, expressed as *S*/*L*, where *S* is the total adsorbent area in the column.**

2. The adsorbent is rigid material impermeable to the analyte and mobile phase components.
3. Adsorbent is characterized by its specific surface area and pore volume, which are evenly distributed axially and radially in the column (this assumption is equivalent to the assumption of column homogeneity).
4. Thermal effects are negligible.
5. The system is at instant thermodynamic equilibrium.

The column void volume, V_0, is defined as the total volume of the liquid phase in the column and can be measured independently.[12] Total adsorbent surface area in the column, S, is determined as the product of the adsorbent mass and specific surface area.

Considering the classical picture of the column slice (Figure 5) with the mobile phase flow rate F and length L packed with small porous particles, the analyte injected at $x = 0$ will be carried through the column with the mobile phase and its concentration in any part of the column is a function of the distance from the inlet x and the time t. The amount of the analyte entering the cross-sectional zone dx during the time dt is equal to $Fcdt$. During the same time dt the amount of analyte leaving the zone dx may be somehow different and can be expressed as $F(c+dc)dt$. The difference in the analyte amount entering zone dx and exiting it at the same time dt will be $Fdcdt$. Note that dc can be positive or negative. The analyte accumulation in the zone dx can be expressed in the form of the gradient of the concentration along the column axis (x):

$$Fdcdt = -F\left(\frac{\partial c}{\partial x}\right)_t dxdt \tag{29}$$

Equation (29) represents the analyte amount accumulated in the zone dx during the time dt. This amount undergoes some distribution processes in the selected zone. These processes are the actual reason for the analyte accumulation. The analyte distribution function in the selected zone is the second half of the mass balance equation – the amount of analyte accumulated in zone dx should be equal to the amount distributed inside this zone. In general form it can be written as

$$-F\left(\frac{\partial c}{\partial x}\right)_t dxdt = \left(\frac{\partial \psi(c)}{\partial t}\right)_x dxdt \tag{30}$$

where the analyte concentration is a function of both the distance and the time and $\psi(c)$ a distribution function which has units of the analyte amount per unit length of the column. This distribution function is the key for the solution of Equation (30) and the definition of this function essentially determines the way how chromatographic retention will be described.

Equation (30) is simplified to

$$-F\left(\frac{\partial c}{\partial x}\right)_t = \left(\frac{\partial \psi(c)}{\partial t}\right)_x \tag{31}$$

This equation states that the formation of the analyte concentration gradient in a fixed moment of time in any place of the column should be equal to the variation of the analyte amount with the time in the same place in the column.

General solution of Equation (30) can be obtained using two classical relationships for partial derivatives (for simple forms of the distribution function $\psi(c)$)

$$\left(\frac{\partial y}{\partial t}\right)_x = \left(\frac{\partial y}{\partial c}\right)_x \cdot \left(\frac{\partial c}{\partial t}\right)_x \tag{32}$$

$$\left(\frac{\partial c}{\partial t}\right)_x = -\left(\frac{\partial c}{\partial x}\right)_t \cdot \left(\frac{\partial x}{\partial t}\right)_c \tag{33}$$

Substitution of Equation (32) into Equation (31) gives

$$-F\left(\frac{\partial c}{\partial x}\right)_t = \left(\frac{\partial \psi(c)}{\partial c}\right)_x \left(\frac{\partial c}{\partial t}\right)_x \tag{34}$$

The partial derivative of the distribution function by the concentration is actually a full derivative since it is independent of the time and position of the analyte in the column as long as the chromatographic system is at the equilibrium. Applying the relationship (Equation (33)) we get

$$F\left(\frac{\partial c}{\partial x}\right)_t = \frac{d\psi(c)}{dc}\left(\frac{\partial c}{\partial x}\right)_t \left(\frac{\partial x}{\partial t}\right)_c \Rightarrow F = \frac{d\psi(c)}{dc}\left(\frac{\partial x}{\partial t}\right)_c \tag{35}$$

The term $(\partial x/\partial t)_c$ is linear velocity of the analyte at concentration c, and can be denoted as u_c.

The final form of the simplified mass balance solution is

$$u_c = \frac{F}{\dfrac{d\psi(c)}{dc}} \tag{36}$$

Since $V_R = FL/u_c$, where L is the column length, Equation (36) can be written in its final form:

$$V_R(c) = L \cdot \frac{d\psi(c)}{dc} \tag{37}$$

Retention volume is essentially proportional to the derivative of the analyte distribution function defined per unit of the column length.

So far the absence of the axial dispersion of the analyte was assumed. Further development of the mathematical description of the chromatographic process requires the definition of the analyte distribution function, or essentially the introduction of the retention model (or mechanism).

VII. PARTITIONING MODEL

As a first example of an applicable model, the traditional partitioning mechanism will be considered. In this mechanism the analyte is distributed between the mobile and stationary phases. Phenomenological description of this process is given in the Introduction section. The V_S and V_M are accordingly the volumes of the mobile and the stationary phases in the column. Instant equilibrium of the analyte distribution between mobile and stationary phases is assumed.

The total amount of the analyte in the column cross-section dx is distributed between the mobile and stationary phases and can be written in the following form:

$$\psi(c) = v_S c_S + v_M c_M \tag{38}$$

where v_S and c_S are the volumes of the stationary and mobile phases per unit of the column length ($v_S = V_S/L, v_M = V_M/L$) and c_S and c_M are the equilibrium analyte concentrations in the stationary and mobile phases, respectively. Since the analyte concentration in the stationary phase is an isothermal function of its concentration in the mobile phase ($c_S = f(c_M)$) using Equation (37) we can write

$$V_R(c_M) = L\frac{d\left[v_S f(c_M) + v_M c_M\right]}{dc_M} \tag{39}$$

If we recall that v_M and v_S are the volumes of the mobile and stationary phase per unit of the column length expression (39) can be converted to

$$V_R(c) = V_M + V_S \frac{df(c)}{dc} \tag{40}$$

where $df(c)/dc$ is the derivative of the partitioning distribution function. For low analyte concentration the distribution function is assumed to be linear and its slope (derivative) is equal to the analyte distribution constant K. Equation (40) then can be written in the well-known form:

$$V_R = V_M + V_S K \tag{41}$$

This equation has been derived for the model of the analyte distribution between mobile and stationary phases and is the same as expression (28) in Section V. To be able to use this equation we need to define (or somehow independently determine) the volumes of these phases.

VIII. ADSORPTION MODEL

An alternative (or just different) description of HPLC retention is based on consideration of the adsorption process instead of partitioning. Adsorption is logically a better model for the description of chromatographic retention, since column packing material consists of solid porous particles with high surface area and is impermeable for the analyte and the eluent molecules.

Adsorbent non-permeability is an important condition, it essentially states that all processes occur in the liquid phase. Since adsorption is related (or retention governed by the adsorption process) to the adsorbent surface it is possible to consider the analyte distribution between the whole liquid phase and the surface. Using surface concentrations and Gibbs concept of excess adsorption it is possible to describe the adsorption from binary mixtures without the definition of adsorbed phase volume.

Adsorption is an accumulation of one component in a close proximity to the adsorbent surface, under the influence of surface forces. In a liquid binary solution, this accumulation is accompanied by the corresponding displacement of another component (solvent) from the surface region into the bulk solution, thus increasing its concentration there. At equilibrium a certain amount of the solute will be accumulated on the surface in excess of its equilibrium concentration in the bulk solution, as shown in Figure 1.

In any instant in the cross-sectional zone dx (Figure 5) of the column, total amount of the analyte could be expressed as

$$n_a = \psi(c) = v_0 c_e + s\Gamma(c_e) \tag{42}$$

where v_0 is the total volume of the liquid phase in that cross-section (the liquid phase volume per unit of the column length), c_e the equilibrium concentration of the analyte in the bulk liquid, s the adsorbent surface area per unit of the column length, and $\Gamma(c_e)$ the amount of analyte excessively adsorbed on the unit of adsorbent surface.

Expression (42) is essentially the analyte distribution function that could be used in the mass balance equation (Equation (31)). The process of mathematical solution of Equation (31) with distribution function (Equation (42)) is similar to the one shown above and the resulting expression is

$$V_R = V_0 + S\frac{d\Gamma(c)}{dc} \tag{43}$$

This expression describes the analyte retention in a binary system using only the total volume of the liquid phase in the column, V_0, and total adsorbent surface area S as parameters and the derivative of the excess adsorption by the analyte equilibrium concentration. For low analyte concentration the slope of the excess adsorption isotherm is constant and expression (43) can be written in the form

$$V_R = V_0 + SK_H \tag{44}$$

where K_H is the Henry constant of the analyte adsorption. It is related to the increment of the analyte retention volume per square meter of adsorbent surface.

Equation (43) is only applicable for binary systems (analyte – single component mobile phase). A similar expression could be derived if we assume that the adsorption of the analyte does not disturb the equilibrium of the binary eluent system. The equilibrium of the binary eluent system is established in the column well before the analyte injection, and two or three orders of magnitude smaller injected quantities of the analyte could be considered as non-disturbing for the eluent adsorption equilibrium in the column.

IX. VOID VOLUME CONSIDERATIONS

In two previous sections of this chapter two alternative theoretical approaches were discussed. In ideal form, for low analyte concentration both adsorption and partitioning retention mechanisms have similar mathematical expressions: Equation (41) for partitioning and Equation (44) for adsorption. The difference is in the parameters describing the geometry of the chromatographic system – stationary phase volume, mobile phase volume, void volume, and adsorbent surface area. Conceptual discussion of all these parameters was briefly given above. In the partitioning mechanism the mobile phase volume could be analyte specific and the definition

of a common volume for all analytes is questionable. The condition of impermeability of the base packing material for the analyte molecules essentially means that total volume of the liquid phase in the column is equal to the sum of the volumes of the mobile and stationary phase in the same column.

The adsorption model of HPLC retention allows clear definition of the column void volume as the total volume of the liquid phase in the column, but use of this model with the use of the surface specific retention and the correlation of the HPLC retention with the thermodynamic (and thus energetic) parameters is not well developed. This model requires the selection of the standard state of a given chromatographic system and relation of all parameters to that state.

In most analytical applications of HPLC all these discrepancies are quietly and conveniently forgotten and usually some components are selected as so called "non-retained" and used as a void volume marker. In the majority of recent analytical publications either thiourea or uracil were used as the void volume markers. As a disclaimer we have to say here that for the purposes of analytical method development, qualitative or quantitative separation of complex mixtures the use of "non-retained" component as void volume marker is fine as long as there are no physico-chemical generalization, or thermodynamic or further theoretical development done on the basis of these results.

As a simple illustration of the inconsistency of any non-retained components for the void volume determination we remind the reader that in the basic assumptions and in the derivation of expressions (41) and (44) void volume of the column was considered to be constant in any eluent type and composition. Figure 6 illustrates the dependence of the thiourea and uracil retention in acetonitrile/water and in methanol/water as a function of organic content in the eluent. The horizontal line in Figure 6 shows the volume of the liquid phase in the column measured according to the procedure described in reference 12. Note, that the retention of both markers for most used eluent compositions is lower than the volume of the liquid phase. This indicates the exclusion of the marker molecules from the adsorbent surface and preferential adsorption of the organic eluent components.

The use of the true volume of the liquid phase in the column as the void volume can lead to the principal difficulties in the interpretation of the retention of polar analytes that are also excluded from the contact with the adsorbent surface. The retention volume of these analytes will be lower than the column void volume and thus their retention factors will be negative. The logarithm of these retention factors does not exist, which shows the limit of the applicability of the approximate theory described above. In a general sense the void volume should not change as a function of solvent type and organic composition.

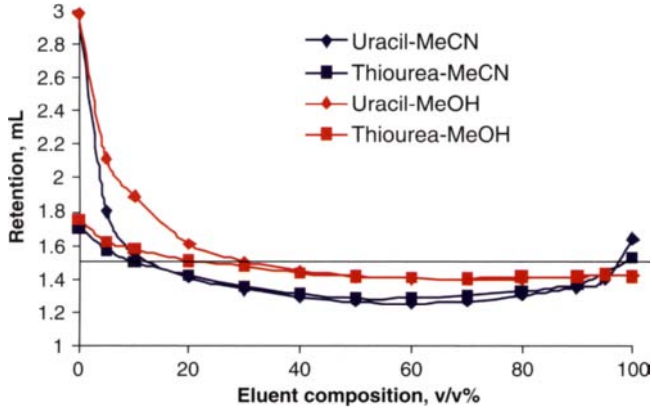

FIGURE 6 **Dependence of the retention of uracil (rhombs) and thiourea (squares) on the eluent composition on Allure-C18 column (150×4.6 mm). Acetonitrile/water (blue lines) and methanol/water (red lines) were used as the mobile phase components. (Reprinted with permission from reference 20.)**

X. THERMODYNAMIC RELATIONSHIPS

The retention (Equation (41)) was derived with the assumption of the analyte partitioning between stationary and mobile phases and partitioning with coefficient K, the thermodynamic function of the Gibbs free energy of the chromatographic system,

$$K = \exp\left(\frac{\Delta G}{RT}\right) \tag{45}$$

Applying this equation to expression (1) we get

$$k = \frac{V_S}{V_0} K \tag{46}$$

which means that the chromatographic retention factor is proportional to the partitioning equilibrium constant (assuming a partitioning retention mechanism with only partitioning equilibrium in the column). The thermodynamic equilibrium constant is the exponent of the Gibbs free energy of the system, so we can write

$$\ln(k) = \frac{\Delta G}{RT} + \ln\left(\frac{V_S}{V_0}\right) \tag{47}$$

Most ideal chromatographic systems with absence of the secondary equilibria effects (such as analyte ionization, specific interactions with active

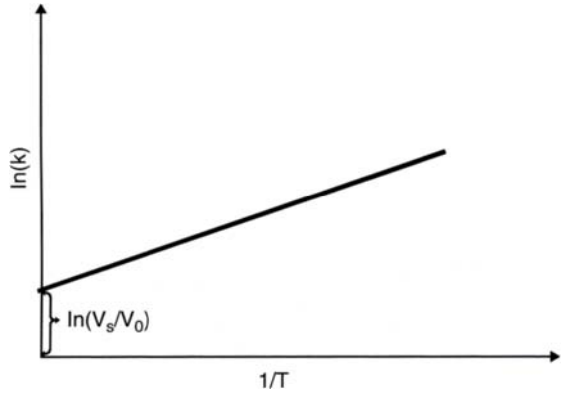

FIGURE 7 Ideal dependence of the HPLC retention on the temperature.

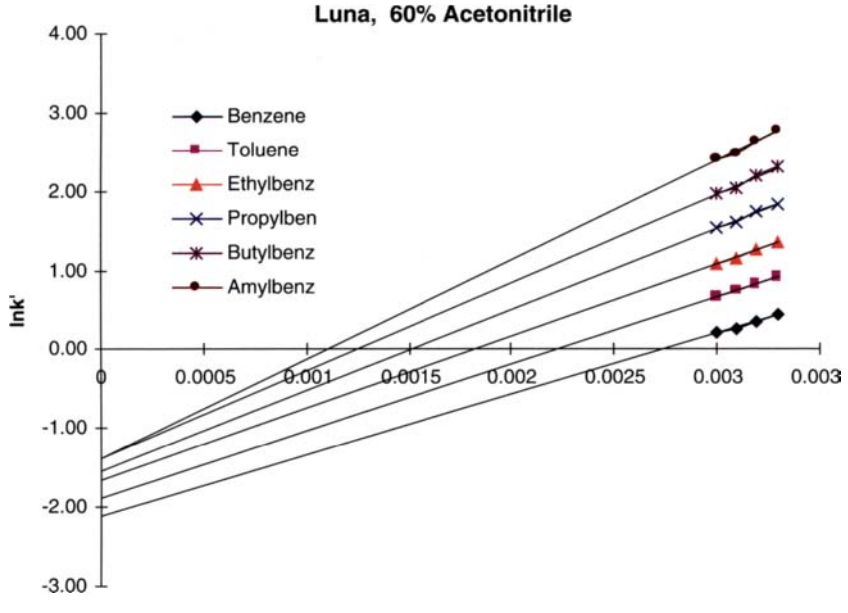

FIGURE 8 Temperature dependencies of alkylbenzenes retention at 60% MeCN/40% Water on Luna-C18 column. (Reprinted with permission from reference 20.)

adsorption sites, etc.) show linear dependencies of the logarithm of the retention factors on the inversed temperature, as shown in Figure 7.

Figure 8 represents the temperature dependencies of a homologous series of alkylbenzenes retention at 60% MeCN/40% Water on a Luna-C18 column, and as can be seen the intercepts for each analyte is different

from others, which essentially means that each analyte requires the determination of its own stationary phase volume (the difference in the intercept is related to the substituents on the nucleus of the molecule and the slope is determined by the nucleus of the molecule).

Liquid chromatography is a competitive process. Analyte molecules compete with the eluent molecules for the retention in the stationary phase, based on that we can define the standard state of chromatographic system as the system without the eluent molecules. Equation (47) can be rewritten in the form:

$$\ln(k) = \frac{\Delta G_{an} - \Delta G_{el}}{RT} + \ln\left(\frac{V_S}{V_0}\right) \tag{48}$$

where ΔG_{an} is the Gibbs free energy of the analyte molecules in the chromatographic system, and ΔG_{el} the Gibbs free energy of the chromatographic system with only the eluent. If we inject a small amount of labeled eluent molecules, $\Delta G_{an} = \Delta G_{el}$, and from expression (48) we get $k = V_S/V_0$, or $V_R - V_0 = V_S$. This result is consistent with the partitioning process, since we only consider the analyte partitioning between the mobile and stationary phases and assume that the eluent is in the mobile phase only.

Effect of the eluent composition could be discussed on the basis of Equation (48). In the simple case of binary eluent (organic/water mixture) as the first approximation we can consider that the Gibbs free energy of the eluent interaction with the packing material surface is a linear function of the eluent composition:

$$\Delta G_{el} = \Delta G_{H_2O} + x\Delta G_{org} \tag{49}$$

where ΔG_{el} is an overall Gibbs free energy of the eluent in the chromatographic system, ΔG_{H_2O} the free energy of water, ΔG_{org} the Gibbs free energy of organic eluent component, and x the concentration of the organic component in the eluent. This consideration assumes an ideal behavior of the system and does not account for adsorption of the eluent components on the surface of the stationary phase. Despite that it gives fairly reasonable description of the effect of the eluent composition on the analyte retention. Substituting Equation (49) in expression (48) we get

$$\ln(k) = \frac{\Delta G_{an} - \Delta G_{H_2O} + x\Delta G_{org}}{RT} + \ln\left(\frac{V_S}{V_0}\right) \tag{50}$$

Equation (50) represents the linear dependence of the logarithm of the retention factor on the eluent composition. It follows that

$$\ln(k) = a + xb \tag{51}$$

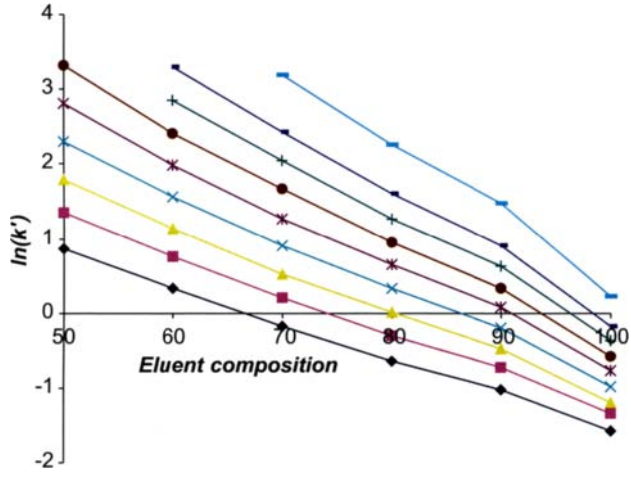

FIGURE 9 Retention of alkylbenzenes (benzene–octylbenzene) on Zorbax-Eclipse XDB C18 column from different compositions of acetonitrile/water eluent. Left pane – retention volumes; right pane – retention factors. (Reprinted with permission from reference 20.)

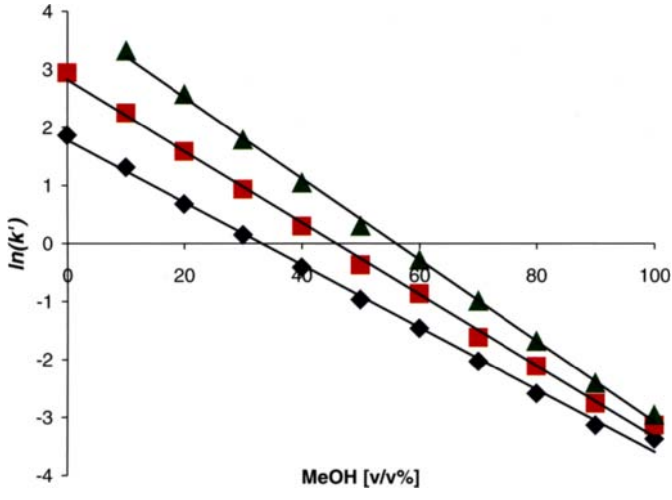

FIGURE 10 Retention of alkylpyridines on Luna-C18 column from methanol/water eluent. (Reprinted with permission from reference 20.)

where a and b are constants. Expression (51) predicts the exponential dependence of the analyte retention on the eluent composition. In most ideal, or close to ideal, chromatographic systems this relationship is fulfilled, as shown in Figures 9 and 10.

This simple dependence of the retention factors on the eluent composition could be used for practical rough estimation of the retention variation with the variation of the concentration of organic modifier in the mobile phase. If the retention of particular analyte was measured at two different eluent compositions, then the retention of that same analyte at any other composition could be roughly evaluated by simple linear extrapolation.

Relationships discussed in this section were derived on the basis of an ideal partitioning model of the chromatographic process. All parameters or energies determined with these relationships are only valid within the frames of this model. A curious consequence of this model is independence of the selectivity on the eluent type and composition, which could be demonstrated as follows.

The selectivity is defined as $\alpha = k_2/k_1$. The same expression could be written as $\alpha = \exp(\ln(k_2) - \ln(k_1))$. Substitution of k' from Equation (48) gives

$$\alpha = \exp\left[\frac{\Delta G_{an.1} - \Delta G_{an.2}}{RT}\right] \tag{52}$$

Expression (52) demonstrates that selectivity is only a function of the analytes' interactions with the stationary phase. It does not include eluent interaction energy meaning that the type and concentration of the organic eluent modifier does not have any influence on the selectivity of partitioning-based chromatographic separation. This effect indeed could be observed for an ideal reversed-phase system, i.e., the separation of benzene and toluene in methanol/water eluent on C18-type column.

XI. SECONDARY EQUILIBRIA

Analyte ionization, tautomerization, or solvation equilibrium in the chromatographic column has a profound effect on the retention and efficiency. These effects are known as secondary equilibria effects.[13] The effect of the analyte ionization on the retention has been extensively studied.[14] Fundamental work by Cs. Horvath et al. created a solid foundation in this field.[15] For ionic equilibria of

$$AH \rightleftharpoons [A^-] + [H^+] \tag{53}$$

with the equilibrium constant expressed as

$$K_a = \frac{[A^-][H^+]}{[AH]} \tag{54}$$

On the basis of the assumption that retention factor is a measure of the stoichiometric mass distribution of the analyte between stationary and

mobile phases, they derive the following expression for the overall retention factor:

$$k = \frac{k_0 + k_1 \dfrac{[H^+]}{K_a}}{1 + \dfrac{[H^+]}{K_a}} \tag{55}$$

where k_0 is the hypothetical retention factor of pure HA and k_1 the retention factor for A^-. This equation represents sigmoidal dependence of the analyte retention on the mobile phase pH with the inflection point corresponding to pH = pK_a (Figure 11), it was first derived by Horvath and Melander.[15]

As we mentioned above, if we define the model of the analyte behavior in the column we can apply this model in the mass balance equation and the solution will be the expression for the analyte retention behavior in the frame of the selected model.

So far the solution of the mass balance equation for models with a single dominating process (partitioning or adsorption) was discussed. In both cases the solutions have similar form with the difference in the definition of the parameters (volumes of the mobile and stationary phases in case of partitioning and total volume of the liquid phase and adsorbent surface area in case of adsorption model).

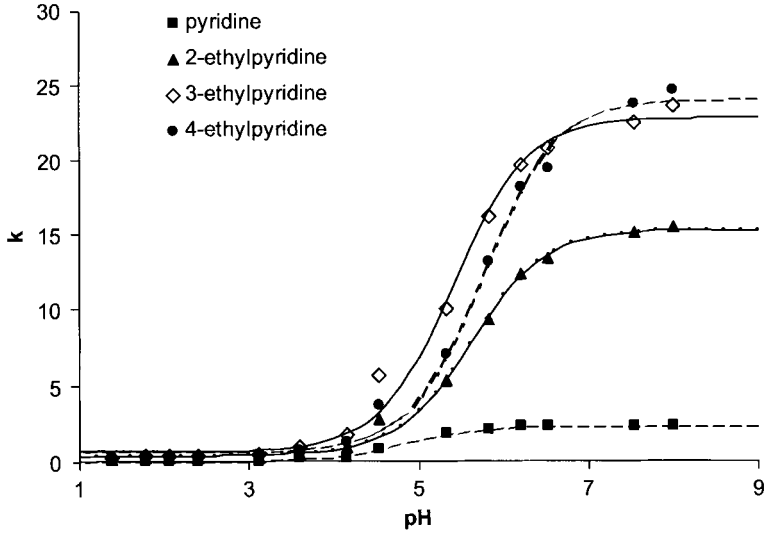

FIGURE 11 Retention of alkylpiridines as a function of mobile phase pH. (Reprinted with permission from reference 16.)

XII. SALT EFFECT

If the counteranion concentration is low, some analyte molecules have a disrupted solvation shell, and some do not, due to the limited amount of counteranions present at any instant within the mobile phase. If we assume an existence of the equilibrium between solvated and desolvated analyte molecules and counteranions, this mechanism could be described mathematically.

The assumptions for this model are:

1. Analyte concentration in the system is low enough that analyte–analyte interactions could be considered non-existent.
2. The chromatographic system is in thermodynamic equilibrium.

The analyte solvation–desolvation equilibrium inside the column could be written in the following form:

$$B_S^+ + A^- \rightleftharpoons B^- \cdots A^- \tag{56}$$

where B_S^+ is a solvated basic analyte, A^- a counteranion, and $B^+ \cdots A^-$ the desolvated ion-associated complex. The total amount of analyte injected is [B], analyte in its solvated form is B_S^+, and analyte in its desolvated form is denoted as $[B^+ \cdots A^-]$, indicating its interaction with counteranions.

The equilibrium constant of reaction (56) is

$$K = \frac{[B^+ \cdots A^-]}{[B_S^+][A^-]} \tag{57}$$

The total analyte amount is equal to the sum of the solvated and desolvated forms of analyte

$$[B] = [B_S^+] + [B^+ \cdots A^-] \tag{58}$$

The fraction of solvated analyte can be expressed as

$$\theta = \frac{[B_S^+]}{[B]} \tag{59}$$

The fraction of the desolvated analyte in the mobile phase can be expressed as

$$1 - \theta = \frac{[B^+ \cdots A^-]}{[B]} \tag{60}$$

Substituting expression (59) and (60) in expression (57) we can write an expression for the equilibrium constant

$$K = \frac{1 - \theta}{\theta[A^-]} \tag{61}$$

Solving Equation (61) for θ solvated fraction) we get

$$\theta = \frac{1}{K[A^-]+1} \tag{62}$$

Expression (62) shows that the solvated fraction of the analyte is dependent on the counteranion concentration and desolvation equilibrium parameter.

Completely solvated analyte has a retention factor (even if it is equal to 0), which we denote as k_s, while the corresponding retention factor for desolvated form is denoted as k_{us}.

Assuming that solvation–desolvation equilibrium is fast we can express the overall retention factor of injected analyte as a sum of the retention factor of solvated form multiplied by the solvated fraction (θ) and the retention factor of the desolvated form multiplied by the desolvated fraction ($1-\theta$), or

$$k = k_s\theta + k_{us}(1-\theta) \tag{63}$$

Substituting θ in Equation (63) from Equation (62) we get

$$k = k_s\left(\frac{1}{K[A^-]+1}\right) - k_{us}\left(\frac{1}{K[A^-]+1}\right) + k_{us} \tag{64}$$

and the final form can be rewritten as

$$k = \frac{k_s - k_{us}}{K[A^-]+1} + k_{us} \tag{65}$$

This equation has three parameters: k_s is a "limiting" retention factor for solvated analyte, k_{us} a "limiting" retention factor for desolvated analyte, and K a desolvation parameter. The resulting function of the protonated analyte retention dependence on the concentration of counteranions is shown in Figure 12 together with experimental retention values.

XIII. EFFECT OF DIFFERENT COUNTERANIONS

The chaotropic theory was shown to be applicable in many situations where small inorganic ions were used for the alteration of the retention of basic pharmaceutical compounds.[18,19] Equation (65) essentially attributes the upper retention limit for completely desolvated analyte to the hydrophobic properties of the analyte alone. In other words, there may be significantly different concentrations of different counterions needed for complete desolvation of the analyte, but its hydrophobicity and thus retention characteristics in completely desolvated form is essentially independent of the type of counteranion employed. Experimental results, on the other hand, show that the use of different

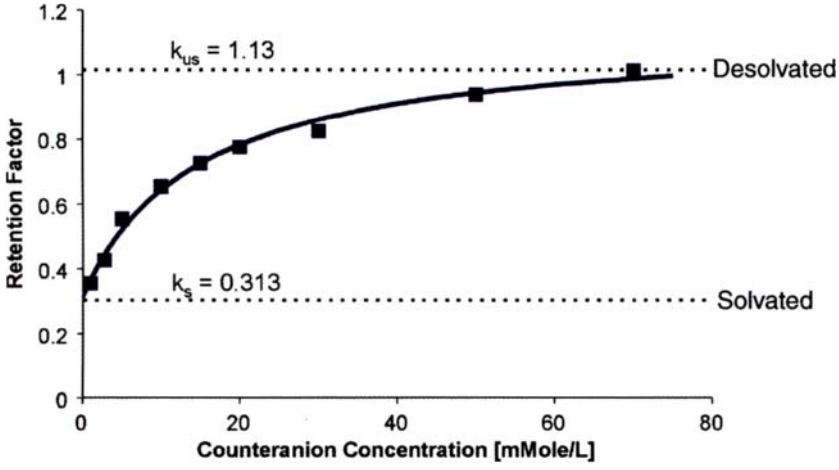

FIGURE 12 Dependence of basic analyte retention on the counteranion concentration in the mobile phase. (Reprinted with permission from reference 17.)

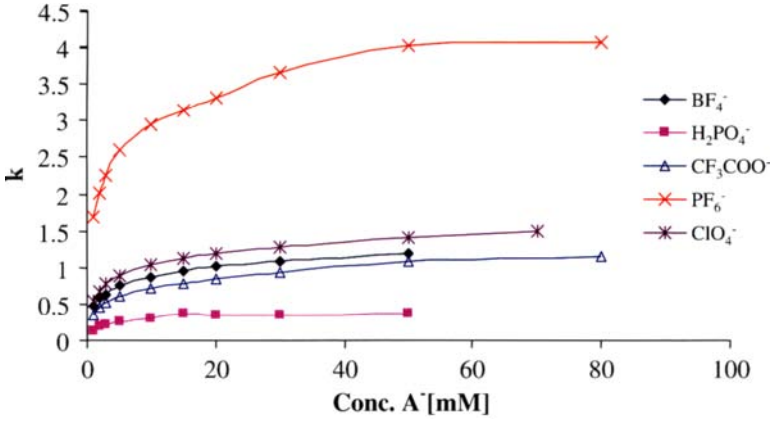

FIGURE 13 Retention factor variations for acebutolol with different chaotropic agents. (Reprinted with permission from reference 18.)

counterions leads to different retention limits of completely desolvated analyte. Figure 13 clearly illustrates this effect.

The effect of different counterions on the chromatographic retention of a β-blockers mixture is demonstrated in Figure 14 where all chromatographic conditions for all four runs are identical, except the type of counterion employed.

This discrepancy essentially suggests the existence of the other process with similar equilibrium and similar effect on the overall retention of protonated basic analytes.

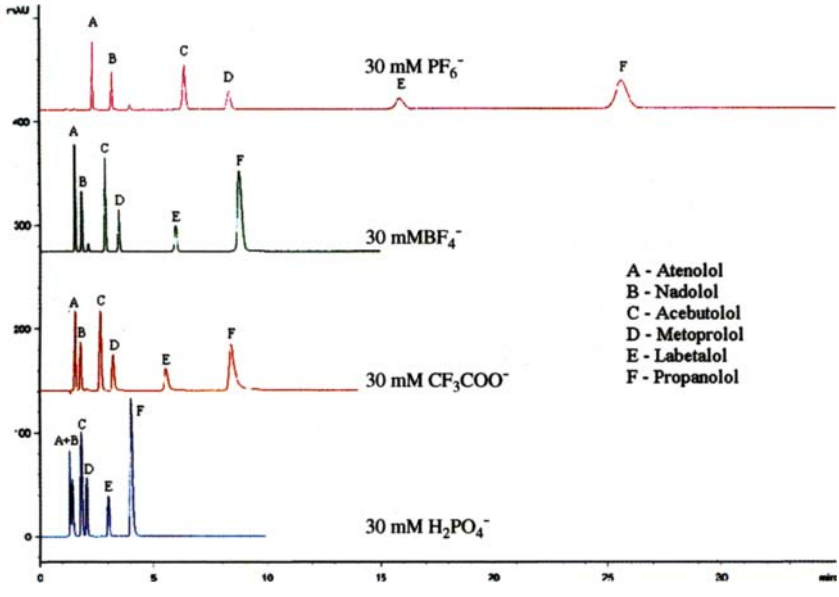

FIGURE 14 Separation of β-blocker mixture with different inorganic anions. Column: Zorbax-Eclipse XDB-C18, Mobile phase (pH 3.0) 70/30 acetonitrile/water at 1 mL/min. (Reprinted with permission from reference 18.)

XIV. INCLUSION OF SECONDARY EQUILIBRIA IN THE MASS BALANCE

In HPLC, analytes could participate in other types of processes besides the adsorption equilibrium, e.g., ionization, tautomerization, solvation, etc. The analyte behavior in adsorption equilibrium is certainly affected by the presence of secondary equilibrium, and if it could be considered as independent of the time and position of the analyte within the column, this equilibrium could be incorporated in the solution of mass balance equation.

Let us assume the presence of ionic equilibrium for ionizable basic analyte

$$B + H^+ = BH^+ \tag{66}$$

The above equilibrium is dependent on the mobile phase pH and the relationship between ionic and non-ionic form of the analyte is described by the Henderson–Hasselbach equation

$$\frac{c_{BH^+}}{c_B} = 10^{pK_a - pH} \text{ or } \frac{c_{BH^+}}{c_B} = \frac{[H^+]}{K_i} \tag{67}$$

where K_i is the analyte ionization constant. If both ionic and non-ionic forms of the analyte can be adsorbed on the surface and if we assume the absence of the ionic equilibrium in the adsorbed state, which means that if the analyte gets adsorbed in the ionic form it stays in this form on the surface.

The left part of Equation (31) expresses the presence of the gradient of the analyte in the column cross-section. In case of ionizable analyte there are two forms of the analyte present and using expression (67) the left part of Equation (31) should be written in the form

$$-F\left(\frac{\partial c}{\partial x}\right)_t dxdt = -F\left(\frac{\partial\left(c_B + c_{BH^+}\right)}{\partial x}\right)_t dxdt = -F\left(1+\frac{[H^+]}{K_i}\right)\left(\frac{\partial c_B}{\partial x}\right)_t dxdt \tag{68}$$

The analyte distribution function in the column cross-section dx (Figure 5) could be described as

$$\psi(c) = v_0 c_B + v_0 c_{BH^+} + s\Gamma_B(c_B) + s\Gamma_{BH}(c_{BH^+}) \tag{69}$$

where v_0 is the volume of the liquid phase in the column per unit of the column length, c_B the equilibrium concentration of the neutral form of basic analyte, c_{BH^+} the equilibrium concentration of the protonated form of basic analyte, s the surface area of the adsorbent in the column per unit of the column length, $\Gamma_B(c_B)$ excess adsorption of the neutral form of the analyte as the function of the equilibrium concentration of corresponding form, and $\Gamma_{BH}(c_{BH^+})$ the excess adsorption of the ionic form as the function of the concentration of this form of the analyte.

Using this distribution function in Equation (31) we get

$$-F\left(\frac{\partial c}{\partial x}\right)_t = \left(\frac{\partial\left[v_0 c_B + v_0 c_{BH^+} + s\Gamma_B(c_B) + s\Gamma_{BH}(c_{BH^+})\right]}{\partial t}\right)_x \tag{70}$$

Equilibrium concentration of the ionic analyte form (c_{BH^+}) in the second term could be substituted with c_B from expression (67) to obtain

$$-F\left(1+\frac{[H^+]}{K_i}\right)\left(\frac{\partial c_B}{\partial x}\right)_t = \left(v_0\frac{\partial c_B}{\partial t}\right)_x + \left(v_0\frac{[H^+]}{K_i}\frac{\partial c_B}{\partial t}\right)_x + \left(s\frac{\partial\Gamma_B(c_B)}{\partial t}\right)_x + \left(s\frac{\partial\Gamma_{BH}(c_{BH^+})}{\partial t}\right)_x \tag{71}$$

and we can use the same transformations of variables as in Equation (34) to get

$$F\left(1+\frac{[H^+]}{K_i}\right)\left(\frac{\partial c_B}{\partial x}\right)_t = \left[v_0\left(1+\frac{[H^+]}{K_i}\right) + s\left(\frac{d\Gamma_B(c_B)}{dc_B}+\frac{[H^+]}{K_i}\frac{d\Gamma_{BH}(c_{BH^+})}{dc_B}\right)\right]\left(\frac{\partial c_B}{\partial x}\right)_t\left(\frac{dx}{dt}\right)_c \quad (72)$$

The expression $(dx/dt)_c$ is the linear velocity of the analyte in the column u_c and, therefore for the final equation for the retention volume of ionizable analyte we get, using the same conversions as in Equations (31–38),

$$V_R = V_0 + S\frac{\left(\frac{d\Gamma_B(c_B)}{dc_B}+\frac{[H^+]}{K_i}\frac{d\Gamma_{BH}(c_{BH^+})}{dc_B}\right)}{\left(1+\frac{[H^+]}{K_i}\right)} \quad (73)$$

At low analyte concentration slope of the excess adsorption isotherm is assumed to be constant and we can substitute the derivatives of the excess adsorption functions for both forms of the analyte with corresponding Henry constants (K_B and K_{BH}):

$$V_R = V_0 + S\frac{\left(K_B+\frac{[H^+]}{K_i}K_{BH}\right)}{\left(1+\frac{[H^+]}{K_i}\right)} \quad (74)$$

At proton concentration at least a hundred times higher than the analyte ionization constant (pH is two units lower than pK_a), expression (74) reduces to $V_R = V_0 + SK_{BH}$. It essentially represents the retention volume of only ionic form of the analyte. At pH at least two units higher than the analyte pK_a (suppressed ionization conditions), expression (74) reduces to $V_R = V_0 + SK_B$ and represents the retention of only non-ionic form of the analyte at conditions where protonation is completely suppressed. Corresponding capacity factors for neutral and protonated forms of basic analyte could be written in the form

$$k'_B = \frac{S}{V_0}K_B \text{ (neutral)} \quad (75)$$

$$k'_{BH} = \frac{S}{V_0}K_{BH} \text{ (protonated)} \quad (76)$$

Substituting K_B and K_{BH} from expressions (75) and (76) into Equation (74) and expressing the overall analyte retention in the form of capacity factor we get

$$k' = \frac{k'_B + k'_{BH}\dfrac{[H^+]}{K_i}}{1 + \dfrac{[H^+]}{K_i}} \tag{77}$$

This is exactly the same expression for the retention of ionizable basic analyte as a function of mobile phase pH which was first derived by Horvath and Melander in 1977.[15]

Equation (74) expresses the retention of ionizable basic analyte as a function of pH and three different constants: ionization constant (K_i), adsorption constant of ionic form of the analyte (K_{BH^+}), and adsorption constant of the neutral form of the analyte (K_B). These three constants describe three different equilibrium processes and have their own relationships with the system temperature and Gibbs free energy of particular analyte form.

XV. CONCLUSIONS

HPLC theory is too broad a topic to be comprehensively covered in one chapter or even in one book. This chapter was intended to create a general understanding of the relationships between different aspects of chromatographic theory, such as analyte retention behavior and retention mechanism.

Participation of the analyte in different processes in the column (ionization, solvation, and other secondary equilibria processes) introduces a complex retention mechanism, which could not be described by a single equilibrium constant, and consequently does not allow facile extraction of representative thermodynamic parameters of intermolecular interactions in the HPLC system.

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3
HPLC COLUMNS AND PACKINGS

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ABSTRACT

In this chapter on HPLC columns, we are discussing both the surface chemistry of a packing as well as column design and performance. In the section that covers column chemistry, we cover modern options of base materials as well as the commonly used approaches towards the surface chemistry of a packing. Specific subsections are dedicated to the selectivity of reversed-phase packings, HILIC, monolithic structures, and the reproducibility of modern packings. In the section on speed and resolution, we familiarize the reader with the principles of how to choose a column. In the section on specialty columns, we cover briefly preparative chromatography and columns with a very small diameter.

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I. INTRODUCTION

When we consider the role of HPLC column technology, we should remind ourselves that the birth hour of true high-performance LC was when it became possible for the first time to pack efficient columns with particles smaller than 30 μm.[1] This improved the separation performance of a standard column from a few hundred theoretical plates to a few thousand, and quite satisfactory analyses could be performed in a time frame of around 15 min to half an hour. This performance resulted in the rapid acceptance of HPLC in the analytical laboratories in the pharmaceutical industry.

An important element of column performance is the particle size of the packing. However, there is much more to the story than just the particle size. We will provide in this chapter the important aspects of column performance in chromatography. In Section II, we will familiarize the reader first with the different options available today with respect to the particle backbone or the structure of chromatographic columns. Then we will look into the very important subject of the surface chemistry of chromatographic packings. What creates the selectivity of the separation? How can this selectivity be characterized in a quantitative way? What are the characteristics of the commonly used types of chromatographic packings? How are they useful in the practice of HPLC? Finally, we will go back to the parameters that make HPLC what it is today: the speed and the resolving power in a separation. How can we assess both in a quantitative way? How can we judge the overall capability of a column? Is there a difference in the case of isocratic chromatography and gradient chromatography, and how can we evaluate column performance under gradient conditions?

In most of these discussions, we will focus on particle-based columns. However, today another option has become available: monolithic columns, both based on silica and on polymeric structures. We will briefly discuss the basic properties of these new structures as well. The most commonly used packings are silica-based packings. Polymeric packings have been an alternative for a long time, but their disadvantages outweighed the advantages, and they are much less frequently used for the HPLC analysis of small molecules. However, polymer-based packings are successfully used in sample preparation techniques. In modern times, other alternatives to silica have been developed, and these newer alternatives have gained a wider acceptance. These include hybrid packings and packings based on zirconia or titania.

In Section IV, we will briefly cover some aspects of unusual column dimensions. Is there any advantage to a column with a very small diameter? What are the aspects of column design and the choice of particle size that are important for preparative applications? This discussion will be brief only.

Of course, all these sections do not need to be read in sequence and stand on their own.

II. COLUMN CHEMISTRY

A. General Comments

All but few particles used in HPLC are fully porous packings. Fully porous packings have a large specific surface area, and this creates a larger retention and a larger loadability, i.e. fully porous packings are less prone to exhibit broad peaks with increased injection (with respect to both volume and mass). A typical specific surface area is in the range of 200–300 m^2/g. This quite sizeable surface area resides in the pores of the packing. The pore size must be sufficiently large to allow access of the analytes to the surface of the packing. For the analysis of small molecules (molecular weight 100–500), a pore size of 10 nm is about right. Some packings have a slightly smaller pore size (around 8 nm), some have a bit larger pores (about 13 nm). One needs to be careful with some of these designations though, since the methods used for the pore size measurements are not consistent from manufacturer to manufacturer, and often only nominal values are given.

The 10 nm (or 100 Å) packings can be used for peptide analysis as well, but for larger molecules (proteins) a larger pore size, nominally 30 nm (or 300 Å), is needed. The larger pore size provides a smaller specific surface area, therefore these larger pore packings are less retentive, and they are not commonly used for small molecules.

The most important properties of a porous particle are the specific surface area and the specific pore volume. "Specific" means that these values are given per gram of packing. This approach is acceptable for a comparison of different silica-based packings, but such values can be misleading, if silica-based packings are compared to zirconia-based packings or polymer-based packings. Chromatographers are usually more interested in which packing would result in more retention or less retention. The retentivity of a particle can be estimated quickly by looking at the ratio of the specific surface area to the specific pore volume. This is called the particle phase ratio β_p:[2]

$$\beta_p = \frac{A_{sp}}{V_{sp}} \tag{1}$$

A_{sp} is the specific surface area (in m^2/g) and V_{sp} is the specific pore volume (in mL/g). However, for a quantitative assessment, the ratio of the surface area of the packing in the column to the column void volume needs to be considered. This true phase ratio inside the column can be calculated as follows:[2]

$$\beta = (1 - \varepsilon_i)\frac{A_{sp}}{V_{sp} + (\varepsilon_i / \rho_p)} \tag{2}$$

ε_i is the interstitial fraction. Its value is typically 0.4 for most HPLC columns. The skeletal density ρ_p is 2.2 g/mL for silica. For polymeric packings, it is around 0.8–1 g/mL. This equation allows for a meaningful comparison of different packings, including non-porous packings.

The advantage of fully porous packings is clear: they have a much larger surface area than non-porous particles. Therefore, their retentivity and loadability is much larger. Equation (2) permits a direct comparison of the retentivity of all packings, whether they are non-porous, porous, superficially porous, or based on silica or another carrier.

It is important to get a true understanding of the basic properties of different particles to obtain a proper assessment of their value in chromatography. You will find that the true phase ratio gives you quite a different impression than the surface area alone. Let us compare two packings! One is a silica packing with a specific surface area of 350 m^2/g and a specific pore volume of 1 mL/g; the other is also a silica packing, but now with a specific surface area of 200 m^2/g and a specific pore volume of 0.5 mL/g. When we calculate the phase ratios, we find that both are practically identical: 178 m^2/mL compared to 176 m^2/mL. Thus, the retentivity and the preparative loadability of both packings should be equal, unless other particle properties affect these values. If we would have used only the specific surface area for judging the retention of these two packings, we would have believed that the one with the larger surface area would be nearly two times as retentive. This comparison points out the importance of Equation (2).

The strength of a particle depends on its specific pore volume. For normal HPLC applications, strength is of no issue, if the specific pore volume is 1 mL/g or less. For ultra-high-pressure applications, a slightly lower pore volume is needed.

B. The Particle Backbone

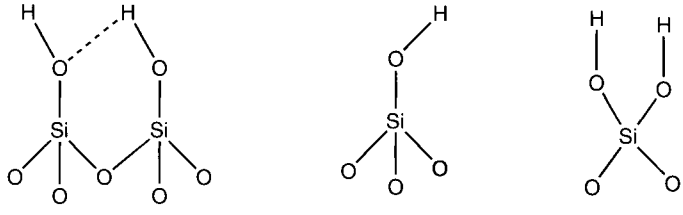
In HPLC, packings based on silica are predominantly utilized. The most important reason for the broad use of silica is its mechanical strength together with the availability of well-established surface modification techniques, especially for the creation of reversed-phase packings (see Section II.C). Another important feature of silica is the freedom with which surface area and pore size can be tailored to specific applications.

One needs to distinguish two types of silica. The classical type is of low purity, and contains a fair amount of other ions in its matrix and on its surface. Common impurities are alumina and iron oxide. Both stem from the raw materials, which are of inorganic origin (water glass). These contaminants are embedded in the matrix of the material, and create acidified surface silanols. Acidic surface silanols are a significant problem when reversed-phase packings based on a classical silica are used in applications common to the pharmaceutical industry, such as the

analysis of compounds containing basic functional groups (amino groups). The classical way to solve the problem is to work only under acidic mobile-phase conditions and/or with bases as competing additives to the mobile phase, or both.

This problem was reduced significantly when high-purity silicas became commercially available. They are synthesized from very pure organic silanes, such as tetraethoxysilane, and metal contamination can be kept out of a silica based on these raw materials by using carefully controlled manufacturing processes. There is a class difference in the peak shape quality for basic analytes between reversed-phase packings based on classical silicas and those based on high-purity silicas. Today, packings based on high-purity silicas are widely available, and there are no compromises that would prevent anyone from using these much higher quality packings based on high-purity silicas. Since method development with high-purity or other modern packings is so much easier, we recommend the use of modern packings for this purpose.

The surface of a silica packing (or a silica-hybrid packing, see below) is occupied by silanols. One distinguishes three types of silanol groups.



bridged or vicinal silanols | lone or isolated silanols | geminal silanols

Lone silanols are more acidic than the other types of silanols, which exist on well-hydrated surfaces. The generally accepted average pK_a of silanols on a fully hydroxylated high-purity silica is around 7.[3] On a low-purity silica, a significant portion of the silanols are more acidic,[4] which is the cause of base tailing on the older silicas.

In recent years, a derivative technology of high-purity silica has been developed: hybrid organic/inorganic packings based on a co-condensation of tetraethoxysilane and other silanes containing silicon–carbon bonds. This technology provides a mixed organic/inorganic matrix. The primary advantage of this technology is an improved stability of the packing to an alkaline environment. Silica-based packings are commonly unstable above pH 8 even at room temperature. A dense coating with a C_{18} layer can alleviate this problem somewhat, but the real solution to this weakness of silica is a modification of the matrix. The incorporation of methyl groups or ethyl bridges into the matrix significantly improves the pH stability of the packing. Methyl-hybrid packings have been reported to be stable above pH 11, while the stability of a newer version, the ethyl-bridged hybrid packing,[4] has been demonstrated to reach pH 12.

A side benefit of at least one version of the hybrid silicas is a shift of the pK_a of the surface silanols into the alkaline pH range.[5] Under the same conditions of measurement, the pK_a of a high-purity silica was measured to be around 7, while it was around 10 for the methyl hybrid packing.[6] This means that the "silanol activity" of the methyl hybrid packing is much reduced compared to silica-based packings, resulting in generally improved peak shapes for basic analytes compared even to high-purity silica packings.

Zirconia-based packings are stable from pH 1 to 14, as well as at elevated temperatures. However, bonding procedures like the silanization of silica are not available for zirconia. For reversed-phase applications, packings are prepared by either a coating of the zirconia with polybutadiene or polystyrene, or by preparing a thin surface layer of carbon, with a subsequent derivatization of the carbon with a C_{18} layer. The zirconia carrier has a large pore size, 30 nm, with an associated lower specific surface area (30 m^2/g). The lower surface area means that commonly 10–30% less organic modifier is needed for getting equivalent retention to a standard silica-based C_{18} column for a neutral analyte.

Due to the unique character of the zirconia surface, one needs to rethink the method development process. Phosphate, bicarbonate, or carboxylic acid buffers are preferred mobile phase additives, but they are also strongly adsorbed to the zirconia surface. The carbon-coated zirconia is more akin to activated carbon than to a C_{18} packing, and it retains some of these characteristics after the C_{18} coating.

Polymer-based packings are very rarely used in reversed-phase applications for small molecules. The reason for this lack of use is, on one hand, the commonly inferior mass transfer properties of polymeric packings (i.e., they give wider peaks), and on the other hand, the swelling and shrinking that goes along with an organic polymer. Polymeric packings are more frequently used in special applications such as size exclusion chromatography, hydrophilic interaction chromatography, large molecular weight biomolecule analysis, or in sample preparation techniques.

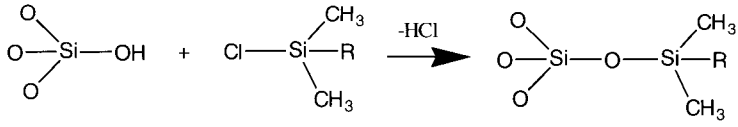
C. The Particle Surface

The particle surface is the most important part of the packing, since it is the interaction with this surface that creates the separation. We will once again focus on silica and silica-hybrid packings since they are most commonly used.

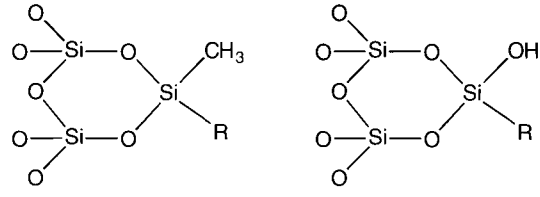
1. Surface Chemistry

The surface silanols of silica are derivatized with organosilanes to create the different types of packings.[7] Typical reagents are chlorosilanes, although other silanes can be used as well. Monofunctional, difunctional, and trifunctional silanes can be equally employed in this surface reaction.

Since HCl is formed during the reaction, a base is used to scavenge the acid. The surface reaction of a monofunctional silane is shown below:

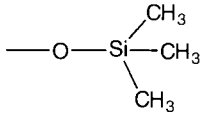


If a difunctional or trifunctional silane is used, more than one bond with the surface is created. This is shown schematically below:



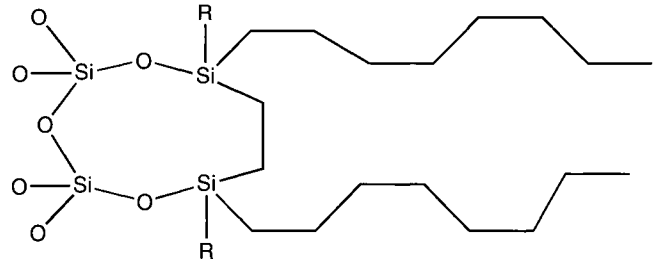
The desired predominant reaction product of a difunctional silane with the surface is the structure shown above. In this way, a maximum number of surface silanols can be removed. However, the details of the actual reaction conditions determine how close one can get to obtaining a large concentration of bidentate bonding. For many bonded molecules, only one link to the surface is formed, and the second silanol is left open.

Trifunctional silanes almost never form a triple link to the surface. Thus, the derivatization with a trifunctional silane creates at least as many new silanol groups as there are ligands attached to the surface. In order to remove these new silanols of multi-functional silanes and residual silanols that were not accessible to the larger monofunctional silanes, the packings are commonly endcapped with trimethylsilyl groups:



This procedure removes additional accessible silanols. However, we must keep in mind that there are about 8 μmol/m^2 of silanols on a fully hydroxylated silica, but the surface reaction and the endcapping can only eliminate about 4 μmol/m^2, leaving still plenty of silanols on the surface. These silanols tend to interact with basic analytes, and may produce peak tailing. This is one of the reasons why hybrid packings have been created, since they exhibit a smaller number of silanol groups on the surface to start with. Modern, high-quality, more sophisticated endcapping techniques can also remove a larger number of these "residual" silanol groups.

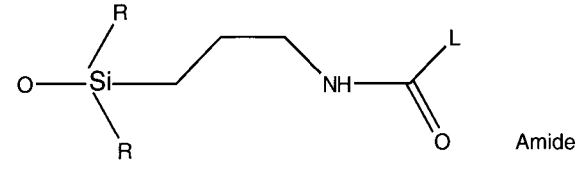
Another bonding option not yet mentioned is the use of bidentate ligands. Such ligands have the capability to attach to the surface in more than one spot. This results in an additional improvement in stability.



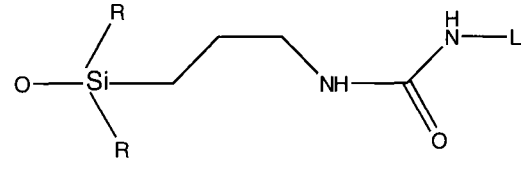
The common side group of monofunctional silanes is a methyl group. However, added stability under acidic conditions can be achieved with more bulky side chains, such as isopropyl or isobutyl groups. The disadvantage of this approach is that the bulkiness of these side chains reduces the surface coverage, and thus more silanols remain and accessible by analyte molecules.

The most common ligand in reversed-phase chromatography is the C_{18} ligand, an 18-carbon hydrocarbon chain. This chain creates the hydrophobic retention of the reversed-phase packing. As an alternative, a C_8 ligand is sometimes used. Due to the shorter chain length, it provides a bit less retention than the C_{18} ligand, but it is occasionally preferred for several reasons. Sometimes, a shorter retention is desired. Some C_8 packings give a slightly improved peak shape compared to a C_{18} ligand, since the smaller ligand can result in a slightly better surface coverage. Or, a slightly different selectivity is desired. It should be pointed out that the selectivity differences between C_{18} and C_8 packings based on the same substrate and with the same surface coverage are rather small, but they still can be useful on occasion.

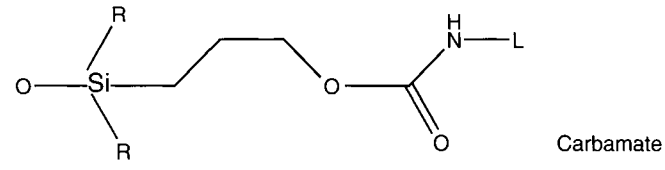
If one is looking for an appreciable difference in selectivity, packings with an embedded polar group are recommended as the first choice. These packings have a polar group incorporated into the long-chain ligand, three carbons away from the silicon group that is used to attach the ligand to the surface.[8] In order to be effective, the polar group is best selected from the group of amide, carbamate, or urea, which all provide excellent hydrogen bonding capability. The three commonly used examples are shown below.



Amide



Urea



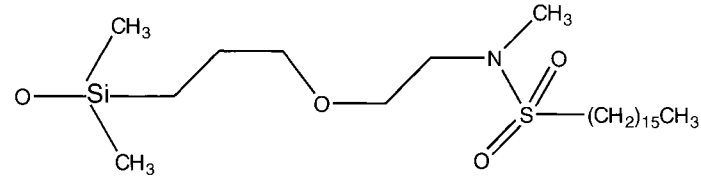
Carbamate

R represents the possible side groups. For a monofunctional ligand (such as the commercially available carbamate phase), R represents a methyl group. Most other packings are based on a trifunctional silane, which means that R is either an additional attachment to the surface or a free silanol.

The length of the ligand varies with the manufacturer. Some short ligands with eight carbons are available, other packings use longer chains, such as a C_{14} ligand.

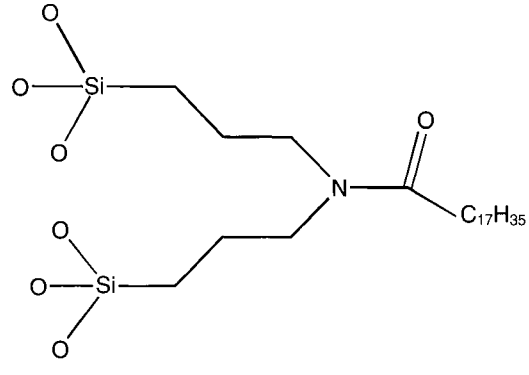
Many (but not all) of the commercially available amide phases are prepared in a two-step reaction, where the silica is first derivatized with an amino silane, and the amide is formed in the second step. This leaves residual amine on the surface. This gives these packings an additional anion exchange function, which can be as detrimental to the peak shapes of acids as silanols are for the peak shapes of bases.

Recently, another packing of the same class was introduced. It is based on a sulfonamide function as the embedded polar group.



This phase does not appear to have the hydrogen bonding ability of the phases mentioned above, but it improves the peak shape for difficult analytes as the other packings with embedded polar functions do.

Finally, a combination of the idea of the bidentate ligand with the idea of an embedded polar group has become commercially available as well. In this case, both attachments to the surface are based on a trifunctional silane, and the distance between "neighboring" attachment groups is rather large:



All packings with an embedded polar group share with each other the fact that they can be used in 100% aqueous mobile phases without dewetting,[9] an improved peak shape for basic analytes, and significantly different selectivity patterns compared to standard C_{18} phases. These differences in the selectivity compared to the classical C_{18} phases can be attributed to the participation of the polar group in the interaction of analytes with the surface of the packing.

Another packing with an embedded polar group is based on a phenol ether. However, this group does not exhibit the characteristic selectivity effects typical of the amide, carbamate, and urea phases.

A comparison of the selectivity difference between different packings is shown in Figure 1. In this figure, the retention times of over 70 analytes measured in an acetonitrile or a methanol gradient at pH 3, 7, and 10 are shown. In Figure 1a, the retention data obtained on a C_8 packing are compared to the same data on a C_{18} packing. In Figure 1b, the same comparison is made for a classical C_8 column and a packing with an embedded carbamate group of the same chain length. One can see that the scatter is much larger for the comparison of the classical column and the embedded-polar-group column (EPG column). This demonstrates the larger selectivity difference created by the embedded polar group.

Figure 2 depicts the details of the selectivity difference between a classical C_8 packing and a packing with an embedded polar group. For simplicity, only the data obtained in methanol are shown. The patterns observed in acetonitrile were similar, but with a smaller impact of the stationary phase selectivity. Generally, the retention is higher for both ionized acids and bases on the classical C_8 packing. However, since the XTerra MS packing is well deactivated, the patterns for the ionic compounds do not differ much from the patterns for general neutral compounds. Analytes with the ability to hydrogen bond to the embedded carbamate group are more retained on the XTerra RP_8 packing. These include phenols, sulfonamides, and non-ionized acids, with the strongest effect for the first two classes of compounds. It should be pointed out that the hydrogen bonding ability of phenols is lost when the phenol group becomes ionized. The same is true for the hydrogen bonding ability of carboxylic acids.

The selectivity differences between EPG packings and classical packings are exciting. However, they have a larger MS bleed than C_{18} packings, which bothers some LC/MS users. The bleed is not due to a larger ligand loss compared to a standard C_{18} packing, but rather due to the much greater ease of ionization of the ligand, which is caused by the embedded polar group. The user needs to decide, if the significant advantages of well-designed EPG packings outweigh this drawback.

Several different types of phenyl ligands are commercially available. They all share a phenyl group, but the attachment to the surface and the length of the chain between the surface and the ligand varies. The common linker is a three-carbon chain, but newer materials have a six-carbon

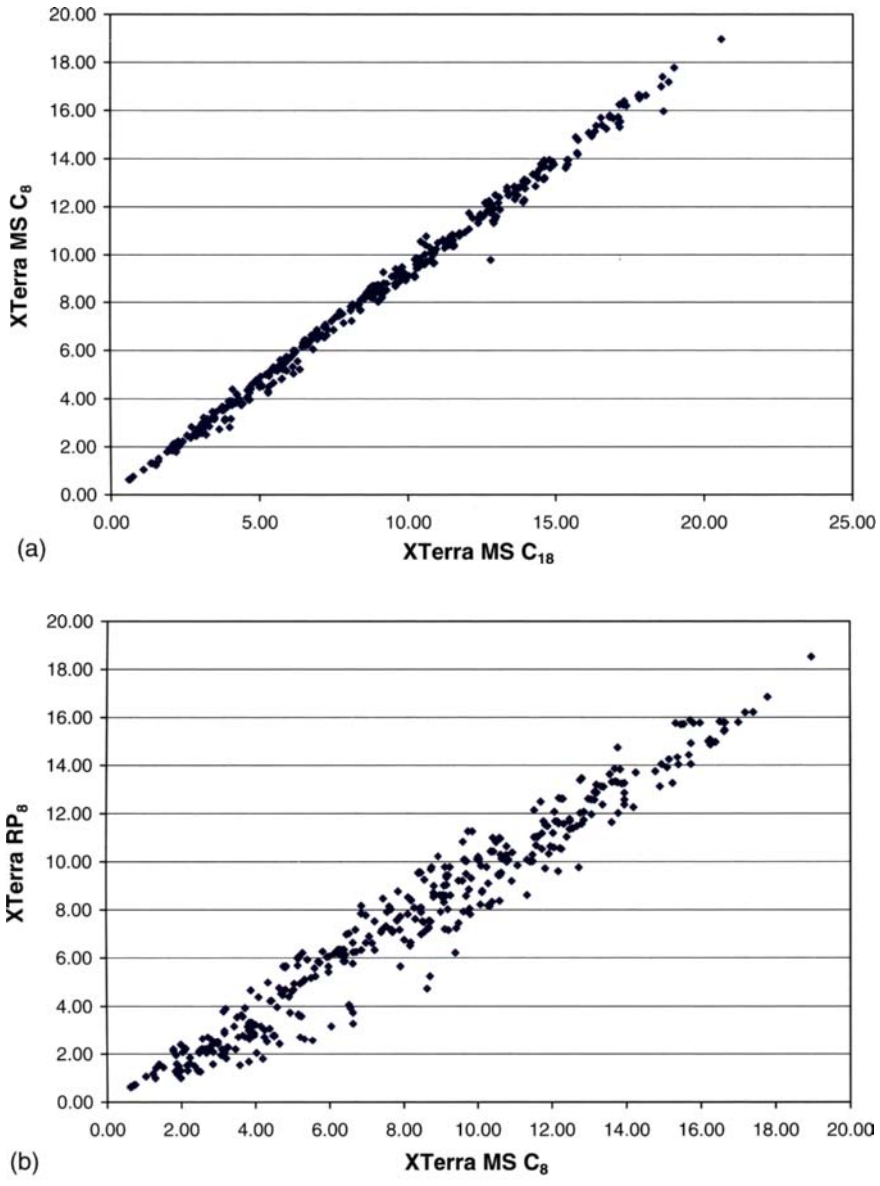


FIGURE 1 Plot of the retention times for over 70 analytes for methanol and acetonitrile gradients at pH 3, 7, and 10 for a change in ligand chain length (a) and a comparison of a packing with an embedded polar group (XTerra RP_8) with one without it (XTerra MS C_8) but with equal chain length (b). The larger scatter for (b) demonstrates the larger selectivity difference between the columns in (b) compared to the columns in (a). Data courtesy of Alberto Méndez, Waters Corporation.

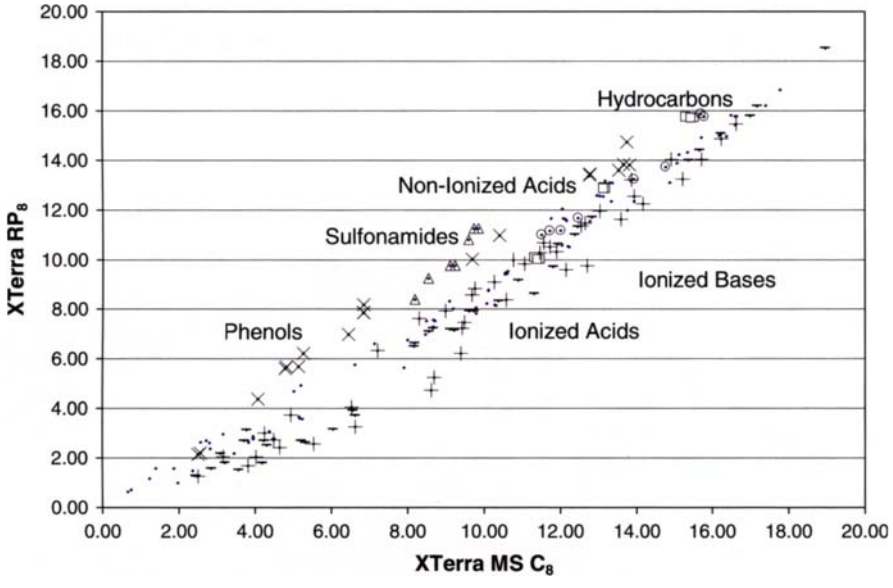


FIGURE 2 **Detailed comparison of the retention pattern of different compound classes on a packing with a classical ligand and one with an embedded polar group. (+) Ionized bases, (−) ionized acids, (×) phenols, (○) non-ionized carboxylic acids, (Δ) sulfonamides. Data courtesy of Alberto Méndez, Waters Corporation.**

spacer (phenylhexyl packings). The phenyl group shows π–π interactions with suitable analytes. Most analytes of interest in the pharmaceutical industry contain aromatic rings and one often sees selectivity differences when switching from a C_8 or a C_{18} packing to a phenyl packing. However, in many cases these selectivity differences are rather small, which actually may be more of a benefit than a detriment. In some special cases, the selectivity and retention differences between a phenylhexyl packing and a C_{18} packing can be striking. Figure 3 shows this for the analysis of a set of explosives standards, carried out on 1.7-μm particles using ultra-high-pressure LC (see also Section III.C). Due to the π–π bonding, the phenylhexyl column exhibits a larger retention and a significant selectivity difference compared to the C_{18} packing.

There are two principal types of perfluorinated packings: one is the aliphatic perfluoro octyl packing and the other one is the aromatic pentafluoro phenyl (PFP) packing. Due to the larger steric hindrance around the more bulky fluorinated groups, the coating levels are usually lower than those for equivalent non-fluorinated packings. As a consequence, both packings exhibit a larger amount of silanol interactions than their non-fluorinated counterparts. Therefore, it is often difficult to determine which selectivity effects stem from the ligand, and which originate in the increased level of silanol interaction. For example, the retention of bases is enhanced on typical PFP packings, which cannot be

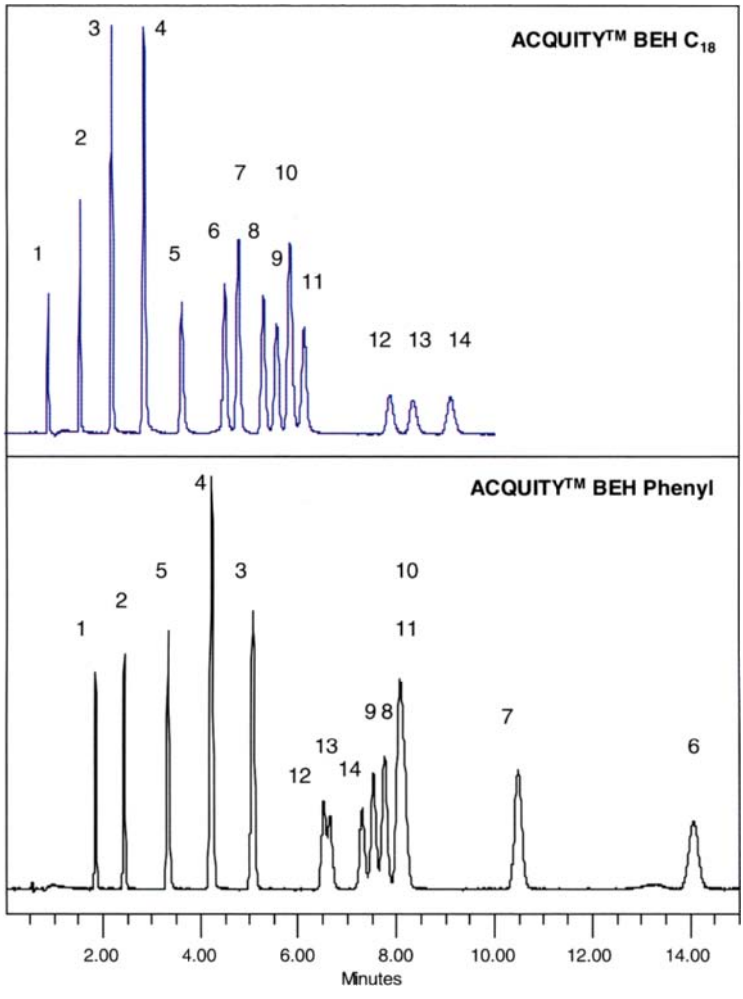


FIGURE 3 **Comparison of the retention of explosives standards on 1.7-µm UPLC packings. Top: 100 mm × 2.1 mm ACQUITY™ BEH C_{18}. Bottom: 100 mm × 2.1 mm ACQUITY™ BEH Phenyl. Flow: 0.5 mL/min. Mobile phase: 28% methanol, 72% water. Instrument: ACQUITY™ UPLC with ACQUITY™ Photodiode Array Detector at 254 nm. Peak designations: (1) HMX, (2) RDX, (3) 1,3,5-trinitrobenzene, (4) 1,3-dinitrobenzene, (5) Nitrobenzene, (6) tetryl, (7) 2,4,6-trinitrotoluene, (8) 2-amino-4,6-dinitrotoluene, (9) 4-amino-2,6-dinitrotoluene, (10) 2,4-dinitrotoluene, (12) 2-nitrotoluene, (13) 4-nitrotoluene, (14) 3-nitrotoluene. Data courtesy of Eric S. Grumbach, Waters Corporation.**

explained readily by a specific interaction with the ligand. Selectivity differences between a perfluoro octyl packing and a standard C_{18} are rather small, except for those that could be attributed to surface silanols.

Cyano (CN) packings have been around since the beginning of HPLC, largely due to their success in GC. However, in LC, many users became

disenchanted with CN packings. This is largely due to multiple stability issues, but not due to a lack of selectivity difference compared to a C_{18}. The standard ligand is a propyl cyano ligand. The attachment to the surface can be done with the use of a monofunctional silane or a trifunctional silane. Due to the shorter chain length and the polar functional group, CN packings are significantly less retentive in reversed-phase HPLC compared to C_8 or C_{18} packings. Due to the difficulties with existing packings, the use of CN packings is not recommended.

2. Selectivity

The general selectivity properties of commercial reversed-phase columns are of significant interest to the applied chromatographer. Several researchers have dedicated large resources to attempt to characterize the main features of commercially available columns.[10–28] Reference 28 (Chapter 4 in this book) describes the approach by one group of authors. The most erudite method and the most inclusive overview of different packings can be found in reference 26, with a recent refinement of the method in reference 27. However, with the exception of the judgment about the hydrophobicity of different packings, the agreement between the different methods is rather poor.[14] The reasons for these discrepancies are not understood. One possibility is that the mobile phase plays a more significant role, and that the dismissal of its influence is the underlying cause for the differences between the characterization methods. From the standpoint of the practitioner, this simply means that one needs to look at the described column features as paintings that have been created with a broad brush, and that one can only extract a general character of a column from the measured values. However, this is still of significant value.

In the following, we will discuss the data collected by us using the method described in reference 13. We have measured the retention of a purely hydrophobic compound, acenaphthene, and that of a compound that shows a strong interaction with surface silanols, amitriptyline, in a methanol/buffer 65.0/35.0 (v/v) mobile phase. The buffer is a 20 mM pH 7.0 phosphate buffer. The logarithm of the retention factor of acenaphthene is the yardstick for the hydrophobicity of the packings. From the logarithm of the relative retention between acenaphthene and amitriptyline, a measure for the activity of the surface silanols is derived. This is demonstrated in Figure 4. In this figure, the silanol activity is plotted versus the hydrophobicity of the packings. C_{18} packings are marked with a black square and C_8 packings with a gray square. Generally, C_{18} packings are more hydrophobic than C_8 packings, but this is not always the case, since the retention for a purely hydrophobic compound depends also on the ligand density and the phase ratio of the parent silica. The circles mark phenyl packings and the diamonds are CN packings. Most phenyl packings are less hydrophobic than C_8 packings, and CN packings are the most polar packings. The stars represent fluorinated packings.

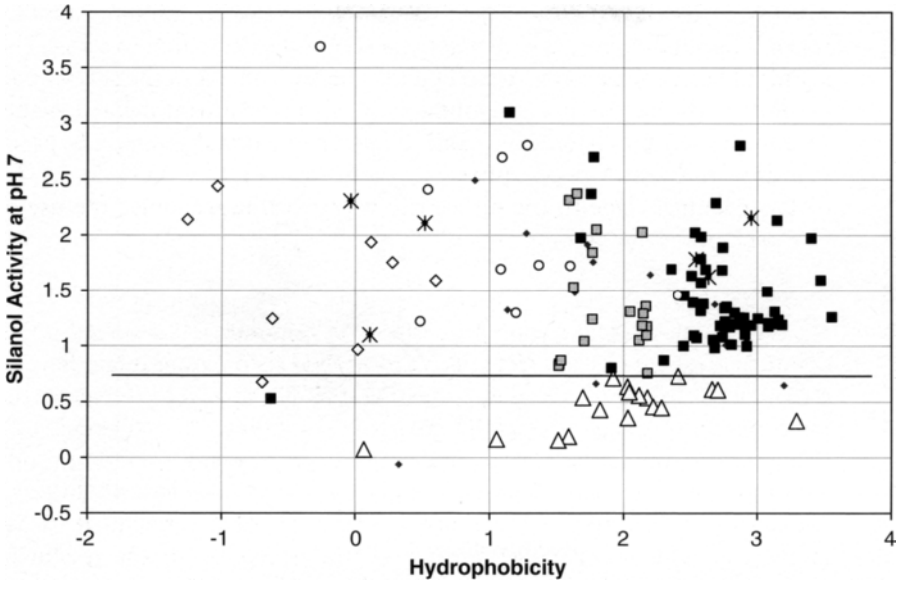


FIGURE 4 **Selectivity chart summary. Designation of packings: black squares: C_{18}, gray squares: C_8, circles: phenyl, diamonds: cyano, stars: fluorinated packings, triangles: packings with embedded polar groups, small back diamonds: uncategorized packings. Reprinted from reference 43.**

The packings marked by a triangle are all packings with an embedded polar group. They clearly show a much lower silanol activity than the packings lacking this feature. This is a general advantage in method development. A line is drawn that shows the difference between packings with an embedded polar group and those packings that lack this feature.

In Figure 5, plots of the values of the silanol activity versus the hydrophobicity are shown for 148 packings. Figure 5a is drawn at the same scale as Figure 4, which was used to orient the reader in the chart. Figure 5b is a magnified view of the indicated section of (a), and (c) is a magnification of (b). The charts cover a broad selection of different packings, from older stationary phases based on low-purity silicas to newer packings based on high-purity silica, to packings based on hybrid particles. The packings were selected from commercial sources in different parts of the world. Occasionally, some packings were tested multiple times to check the reproducibility of the packings. Therefore, the chart contains more data points than packings.

Some packings with a very high silanol activity such as the Zorbax Classic or the Resolve C_{18} were not included in this chart due to the extremely high silanol activity. The same holds true for unendcapped C_{30} packings such as the YMC C_{30}. Among the remaining packings, the highest silanol activity on the chart is found for Waters Spherisorb Phenyl (#4), followed by Platinum EPS (#18). Low silanol activity is

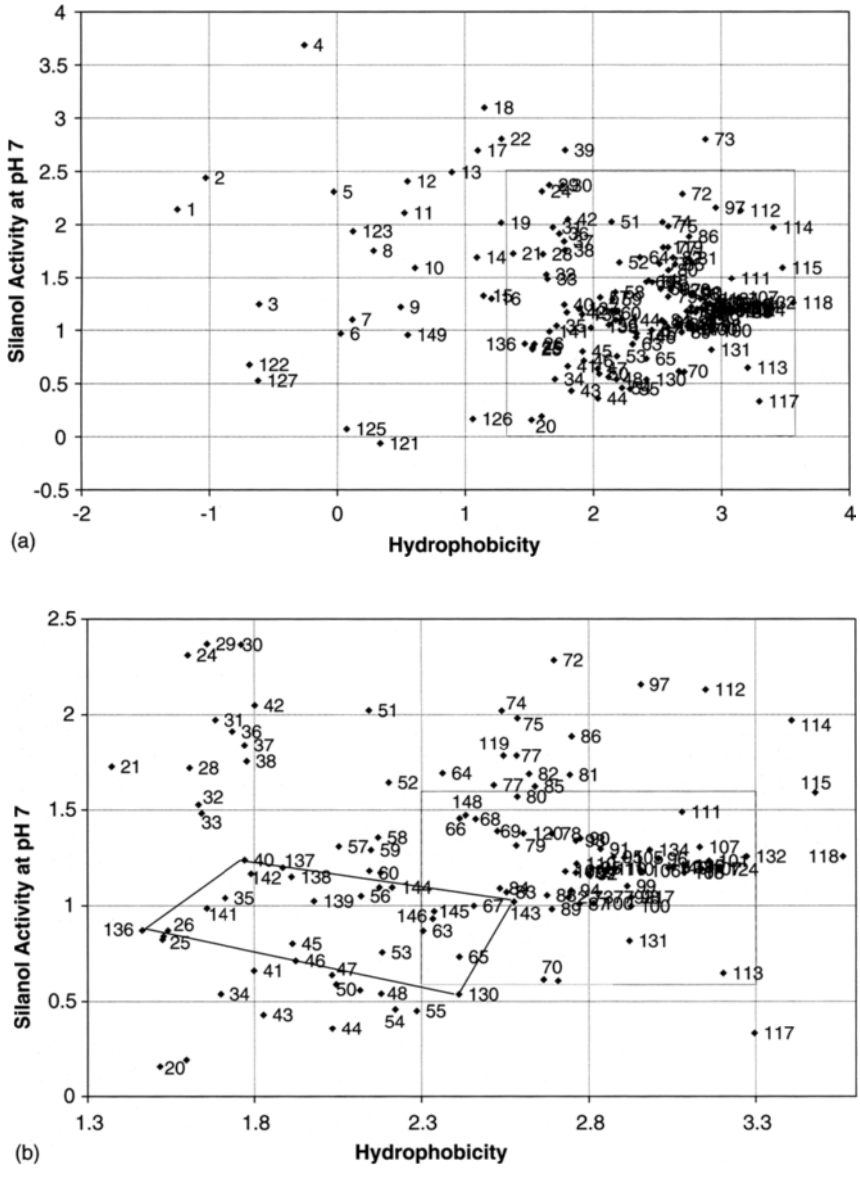


FIGURE 5 (a) Column selectivity chart. (b) An expansion of (a), as indicated, and (c) is an expansion of (b). The same column designations are used in (a)–(c). (1) Nova-Pak CN HP, (2) Waters Spherisorb CN RP, (3) Hypersil CPS CN, (4) Waters Spherisorb Phenyl, (5) Keystone Fluofix 120N, (6) YMC-Pack CN, (7) Ultra PFP, (8) Zorbax SB-CN, (9) Hypersil BDS Phenyl, (10) Inertsil 3 CN, (11) Fluophase RP, (12) Hypersil Phenyl, (13) Zorbax SB-Aq, (14) YMC-Pack Ph, (15) YMC Basic, (16) Ultra Phenyl, (17) Inertsil Ph3, (18) Platinum EPS C_{18}, (19) Synergi Polar-RP, (20) XTerra RP_8, (21) Nova-Pak Phenyl, (22) Zorbax SB-Phenyl, (24) Zorbax Rx C_8, (25) XTerra MS C_8, (26) Prodigy C_8, (28) Zorbax Eclipse XBD Phenyl, (29) Zorbax SB C_8, (30) μBondapak C_{18},

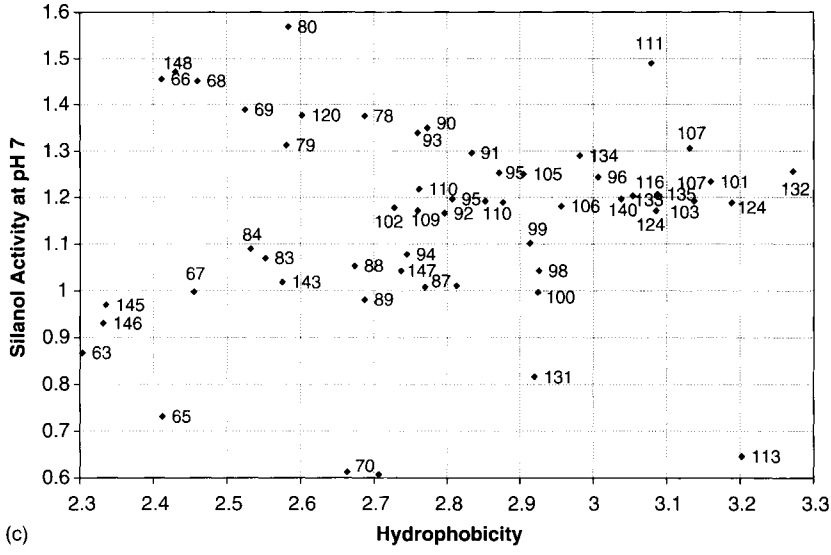


FIGURE 5 Cont.

(31) YMC J'Sphere L80, (32) Supelcosil LC DB-C_8, (33) ZirChrom PBD, (34) Discovery RP Amide C_{16}, (35) Hypersil BDS C_8, (36) HydroBond AQ, (37) Lichrospher Select B, (38) Allure Ultra IBD, (39) Platinum C_{18}, (40) Nova-Pak C_8, (41) Capcell Pak C_{18}, (42) Alltima C_8, (43) Discovery RP AmideC$_{16}$, (44) XTerra RP_{18}, (45) Symmetry300 C_{18}, (46) Spectrum, (47) Zorbax Bonus RP, (48) Supelcosil LC-ABZ Plus, (50) SymmetryShield RP_8, (51) Lichrosorb Select B, (52) PolyEncap A, (53) Prism, (54) Supelcosil LC-ABZ+, (55) Supelcosil LC-ABZ, (56) Luna C_8 (2), (57) Inertsil C_8, (58) Kromasil C_8, (59) Zorbax Eclipse XDB C_8, (60) Symmetry C_8, (63) Hypersil HyPurity Elite C_{18}, (64) Hypersil ODS, (65) Polaris C_{18}-A, (66) Luna Phenyl-Hexyl, (67) Hypersil BDS C_{18}, (68) Supelcosil LC DB-C_{18}, (69) Aqua C_{18}, (70) SymmetryShield RP_{18}, (72) Nucleosil C_{18}, (73) Waters Spherisorb ODS-2, (74) Waters Spherisorb ODSB, (75) YMC J'Sphere M80, (77) Zorbax SB-C_{18}, (78) Synergi Max RP, (79) YMC Hydrosphere C_{18}, (80) Nova-Pak C_{18}, (81) PolyEncap C_{18}, (82) TSK-Gel 80Ts, (83) Ace C_{18}, (84) XTerra MS C_{18}, (85) Fluophase PFP, (86) Purospher RP_{18}, (87) Develosil C30 UG 5, (88) Develosil ODS UG 5, (89) Hypersil Elite C_{18}, (90) Zorbax Rx C_{18}, (91) Zorbax Eclipse XDB C_{18}, (92) L-Column ODS, (93) YMC ODS AQ, (94) Prodigy C_{18}, (95) Luna C_{18} (2), (96) Kromasil C_{18}, (97) Allure PFP Propyl, (98) Discovery HS C_{18}, (99) Inertsil ODS-2, (100) Symmetry C_{18}, (101) L-column ODS, (102) Puresil C_{18}, (103) Cadenza CD-C_{18}, (105) Luna C_{18}, (106) Zorbax Extend C_{18}, (107) Inertsil ODS-3, (109) Zorbax Eclipse XDB C_{18}, (110) YMC Pack Pro C_{18}, (111) Purospher RP_{18}e, (112) Alltima C_{18}, (113) ODPerfect, (114) YMC J'Sphere H80, (115) Develosil ODS SR 5, (116) Nucleodur Gravity C_{18}, (117) Inertsil ODS-EP, (118) YMC-Pack Pro C_{18} RS, (119) Discovery HS F5, (120) Atlantis dC$_{18}$, (121) Discovery HS PEG, (122) Discovery Cyano, (123) Luna CN, (124) Cadenza CD-C_{18}, (125) experimental carbamate CN packing, (126) experimental carbamate phenyl packing, (127) Imtakt Presto FT C_{18}, (128) Aquasil C_{18}, (129) Pursuit Diphenyl, (130) XBridge Shield RP_{18}, (131) Acclaim PA, (132) Inertsil ODS 3V, (133) Capcell Pak MGII 5 µm, (134) Capcell Pak MGII 3 µm, (135) Acclaim C_{18}, (136) XBridge C_8, (137) ACT Ace C_8, (138) ACT Ace C_{18}, (139) Hypersil Gold, (140) SunFire C_{18}, (141) XTerra Phenyl, (142) XBridge Phenyl, (143) XBridge C_{18}, (144) SunFire C_8, (145) Pursuit C_{18} 3 µm, (146) Discovery C_{18}, (147) Gemini C_{18}, (148) Synergi Fusion RP. Data supplied by Bonnie Alden.

shown for all the types of packings with embedded polar groups. The polyethylene-glycol-derivatized Discovery HS PEG (#121) marks the bottom of the chart.

The most hydrophobically retentive packing on the chart is YMC-Pack Pro RS combined with a silanol activity typical of high-purity silicas (#118). Intermediate hydrophobicity for a C_{18}-type packing is exhibited by XTerra MS C_{18} (#84). The most polar stationary phase on the chart is the Nova-Pak CN HP packing (#1). The reader is encouraged to examine the three selectivity charts in detail to find the positions of his or her favorite packings.

Today, many automated method development schemes use a parallel exploration of the selectivity of different columns. Based on the characteristics of the packings described here and in the previous paragraph, we have recommended to use a classical C_8 or C_{18}, a short-chain or long-chain EPG column, and a phenyl column together with the selectivity of the mobile phase to search for a quick start in method development.[29] A typical scheme would use one of each type of columns, methanol and acetonitrile as solvents, and, if the analytes are ionizable, pH as the primary tools in the exploration of separation selectivity. Subsequently, mobile phase composition and temperature can be used for a method fine-tuning. Such a scenario uses every one of these principal selectivity parameters with maximal effect.

These different selectivity characteristics can even be found within a given family of packings. The advantage of such an approach lies in other common factors of the packings. For example, the family of XBridge packings exhibits an excellent chemical stability at alkaline pH. At the same time, the character of the surfaces is measurably different. We can find the packings of this family on Figure 5b (connected lines): XBridge C_8 (#136) is found on the left side of the graph. The most hydrophobic packing in this family is XBridge C_{18} (#143). The packing with the lowest silanol activity is the one with the incorporated polar group XBridge Shield RP_{18} (#130). The XBridge Phenyl packing (#142) has an intermediate hydrophobicity, but also shows selective π–π interaction typical of the phenyl packings (see Section II.C.1). When combined with mobile phase selectivity, these different stationary phase characteristics maximize the chances that a suitable separation will be found rapidly.[29]

3. Other Techniques: HILIC and Mixed-Mode Phases

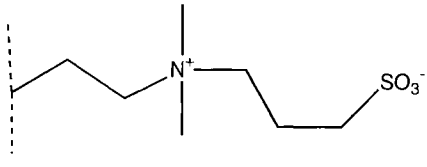
In the majority of this chapter, we are focusing on reversed-phase chromatography, since it is the most commonly used technique in the pharmaceutical industry. However, some alternative techniques may be of advantage under appropriate circumstances. One of these techniques is hydrophilic interaction chromatography, commonly abbreviated as HILIC. Like reversed-phase chromatography, aqueous–organic mobile phases are used. However, the stationary phases are very polar, and thus the overall retention patterns are opposite to what is found with

reversed-phase chromatography. The most common organic modifier is acetonitrile, and retention increases with an increase of acetonitrile in the mobile phase, and decreases with the addition of water. A good overview of the application of this technique for bioanalytical LC/MS can be found in reference 30.

The basic mechanism of HILIC is partitioning of the analytes from the acetonitrile-rich mobile phase to the water-rich surface of the packing.[2] In other words, more polar compounds such as sugars are well retained, while hydrophobic compounds such as toluene are unretained and can serve as void-volume markers. In addition, some of the commercial packings used for HILIC also have ion-exchange properties, which can be advantageous for improving the separation or altering retention. It should be mentioned here that HILIC methods have been demonstrated to yield superior sensitivity in applications where electrospray mass spectrometry is used as the detection technique.[30] The improvement in sensitivity is often one order of magnitude, and may occasionally reach much higher values.[31]

The classical HILIC application is the separation of sugars on silica-based propylamino columns.[32] Due to stability issues with a standard amino column, modern "carbohydrate columns" are stabilized to overcome this difficulty. Also, polymer-based amino columns are available for this application. Due to the amine function, these columns can also be used in a combination of HILIC with an anion-exchange mechanism. Underivatized silica columns can also be used for HILIC applications. In this case, the polar surface of the silica is responsible for the attraction of the water from the mobile phase. In the case of silica, the HILIC retention mechanism is supported by cation-exchange with the surface silanols.[31] It needs to be mentioned that in HILIC, silanols are not a detriment. The tailing observed with silanol-rich packings in reversed-phase chromatography is due to the fact that the silanols are few in number and buried under the hydrophobic C_{18} layer. In HILIC, the silanols are on the surface and easily accessible. Therefore, they can interact freely and without steric hindrance with the analytes. Thus, tailing is observed only rarely.

A modern development in HILIC are zwitterionic stationary phases. The structure of the group responsible for the retention is shown below:



Phases with such groups can be prepared on silica and on organic polymers, and both forms of this packing are commercially available. Note that the opposite charges balance each other. The retention is created by the preferential binding of water by the charged groups. These packings work equally well for anionic, cationic, and neutral analytes.

Another recent development of interest is the use of mixed-mode stationary phases, where hydrophobic interaction is combined with an ion-exchange mechanism. In these stationary phases, ion-exchange groups are incorporated into a reversed-phase ligand. Several different combinations of this type are commercially available, combining strong and weak cationic and anionic functions on the surface with reversed-phase ligands. Details about the structure of the ligands are unfortunately not available.

Mixed-mode stationary phases on the basis of polymeric packings have been used extensively in sample preparation.[33] In this case, a packing prepared from divinylbenzene and *N*-vinyl pyrrolidone is partially derivatized with sulfonic acid groups, carboxylic acid groups, quaternary ammonium groups, or tertiary amino groups to create strong and weak cation and anion exchangers. Such packings interact both via the ion-exchange groups as well as via the hydrophobic matrix (divinylbenzene). Applications include the sample preparation of plasma and urine samples prior to HPLC analysis, often in combination with MS detection. Generally, it was found that techniques using the mixed-mode packings resulted in cleaner HPLC samples with significantly less ion-suppression in MS.

Occasionally, good sample preparation techniques can be combined in an optimal way with HILIC as the analytical technique prior to MS detection. Next, we will describe an interesting application that combines the sample cleanup with the column chemistry and the detection. The ionizable analyte in a (rat) plasma sample is cleaned up via solid-phase extraction using a mixed-mode ion-exchange technique. The elution solvent in this technique is methanol to eliminate hydrophobic interaction, together with an acid or a base to break the ion-exchange interaction.[33] This sample can subsequently be injected directly (i.e. without evaporation to dryness and reconstitution in another solvent) onto a HILIC column. Under HILIC conditions, methanol is a weak eluent and therefore a rather large injection volume is possible. Retention is further enhanced for basic compounds, if the HILIC column is a silica column, since the ion exchange with surface silanols assists the HILIC process. If the detection method is ESI-MS, one can achieve a higher sensitivity in mobile phases that are rich in organic solvent, as is typical for a HILIC method.[30] Such a strategy takes full advantage of the sample preparation, column chemistries, and detector sensitivity available.

D. Monolithic Structures

Since their inception in the mid-1990s, monolithic structures have inspired the imagination of chromatographers. However, the practice has remained far behind these early expectations.

What are monoliths? They are continuous structures of the stationary phase, with a continuous macropore structure that provides the channels for the flow of the mobile phase.[34] The stationary phase can be

porous or non-porous, with the same drawbacks of a non-porous structure as exhibited by non-porous beads in packed beds. In standard monoliths, the internal pore structure, also called micropores, provides the surface area necessary to achieve adequate retention in chromatography.

Monoliths are available based on both silica[35] and organic polymers.[36] Most of the following discussion will focus on silica monoliths.

With packed beds, the volume of the interstitial channels between the particles is typically 40% of the column volume (it is a bit higher for irregular-shaped particles, and it may also be somewhat lower for spherical particles, depending on the properties of those particles). The size of these interstitial channels determines the backpressure of the column, while the size of the particles determines the column performance. With a fixed interstitial volume fraction, the column performance and the column backpressure are simultaneously determined by the particle size in packed beds.

With monoliths, this link no longer exists. Thus, it is possible to create structures with a larger interstitial fraction combined with a smaller characteristic size for the stationary phase than would have been possible with a packed bed. Since the backpressure depends inversely to roughly the fifth power on the interstitial fraction (in the range of 40% interstitial fraction), one can gain significantly by creating a more open monolithic structure. On the other hand, new methods for achieving a high level of macroscopic structural uniformity could lead to further improvements.

The analysis of the performance of standard commercial monoliths[37] showed that the chromatographic performance (van Deemter A-term) of the devices examined was similar to a well-packed bed of 3.5–5-μm particles, while the backpressure was equivalent to that of a column packed with 8–10-μm particles. This gives a clear impression of the performance advantage of a standard monolith. At the same time, the pressure that can be applied to a commercial monolith is limited by the fact that the encasement of the column is made out of PEEK™, which limits the pressure that the column can be exposed to (and thus the speed of analysis that can be achieved). Commercial silica monoliths are still limited to a single internal diameter. Within these constraints, good performance can be achieved.

Organic monoliths are commercially available as well, but they are primarily used as media in the separation of large molecular weight compounds, mostly biomolecules. Typical applications include proteins, peptides, or DNA fragments. A study of the operational variables in such systems has been published.[38]

E. Reproducibility

In the pharmaceutical industry, the reproducibility of a separation is of utmost importance. Once a separation has been developed,

it is expected that it can be transferred to other departments (e.g. QC department) or to other locations, often in other parts of the world, without any difficulties. The method developers also count on the stability of the method over long periods of time, i.e. decades. Both of these subjects have several implications.

First of all, after the completion of method development, it is very important to test the reproducibility of a method from instrument to instrument, and from column to column. With respect to the column reproducibility, it is also worthwhile to compare used and new columns, and columns containing different preparations (=batches) of the packing material. Major column manufacturers can supply such columns, and some have such column sets in their standard offering. The comparison of a used column and a brand-new column ensures that the method is indeed reproducible. The comparison between columns from the same batch of material should give essentially identical results. The comparison between different preparations of the packing material gives an indication of the long-term reproducibility of a method. Good results increase the comfort of the QC department that the method will be stable over long periods of time.

Second, the choice of columns used in method development needs to take into consideration the distribution capability of the column manufacturer. Many pharmaceutical companies have manufacturing sites in different parts of the world, and the same column needs to be readily available in different countries.

The third point is the capability of the column manufacturer to ensure a long-term supply of the packing. This is more likely, if the column supplier is an integrated manufacturer. This means that the column manufacturer synthesizes the raw silica *and* does the surface bonding and column manufacturing. Some column manufacturers do not have the capability to synthesize the raw silica. Thus, they depend on another supplier to provide them with the silica. This has consequences for the security of the supply, and potentially, also the reproducibility of the final product.

The quality of the batch QC-test of the column manufacturer together with reproducibility data of a packing material can also be taken into account in the selection of columns deemed for the development of QC procedures. Such data have, for example, been published in reference 39. Data on the long-term reproducibility of packings are also available from manufacturers upon request. An example of a dataset on the long-term reproducibility of one packing is shown in Figure 6. Relative retention values are plotted versus the batch numbers of all released batches of the product. The range of each retention window is $\pm 10\%$ around the mean value. Let us discuss briefly the different values measured and the meaning of these values. The simplest value is the hydrophobic selectivity measured by the relative retention between

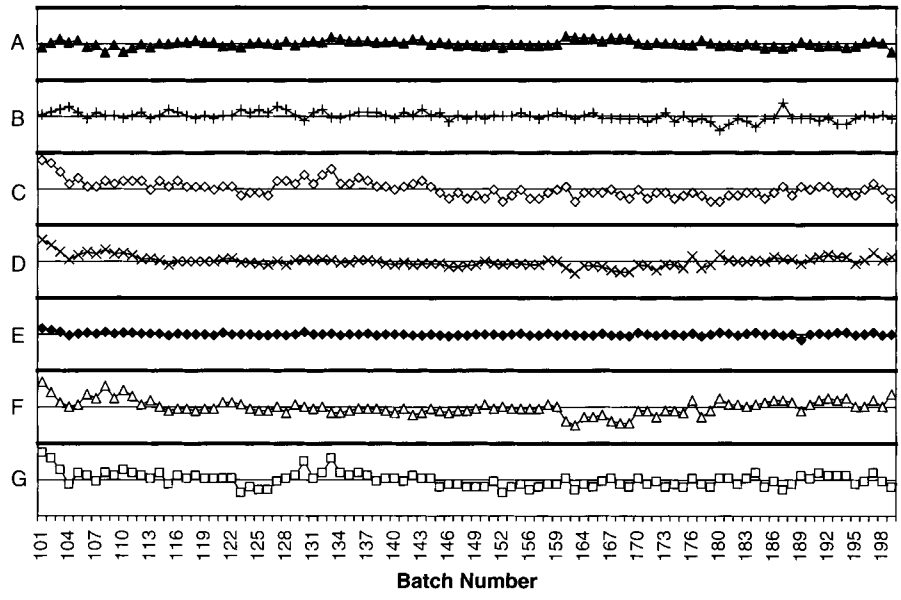


FIGURE 6 **Batch-to-batch reproducibility of the Symmetry C_{18} packing. Range of each value: ±10% around the mean. (A) ▲: α-dipropylphthalate/butylparaben, (B) +: α-amitriptyline/propranolol, (C) ◊: α-amitriptyline/acenaphthene, (D) ×: α-dipropylphthalate/acenaphthene, (E) ●: α-naphthalene/acenaphthene, (F) Δ: α-butylparaben/acenaphthene, (G) □: α-propranolol/acenaphthene. The chart includes all data of the released product since the introduction of the packing. Data supplied by Ed Grover, Waters Corporation.**

acenaphthene and naphthalene (Figure 6E). This value has a relative standard deviation of $<0.4\%$. As discussed above, the relative retention between a base and a neutral purely hydrophobic reference compound is a measure of the silanol group activity of a packing (Figure 6C and G). The test is carried out at pH 7.0, where the partial ionization of the surface silanols critically influences the retention of the positively charged base. The standard deviations of the relative retention of both bases to acenaphthene are around 2%. The relative retention between both bases (Figure 6B) is a measure of access to the surface silanols: one base is a secondary amine and the other a tertiary amine. The standard deviation of this value is 1.2%. The relative retention values between dipropylphthalate and acenaphthene (Figure 6D) and between butylparaben and acenaphthene (Figure 6F) reflect hydrogen bond acceptor and hydrogen bond donor activity of the surface. The first parameter has a standard deviation of 1.5% and the second one a value of 2.0%. The relative retention between the two polar compounds dipropylphthalate and butylparaben (Figure 6A) is a direct measure of the hydrogen bonding capability of the packing (without reference to hydrophobic interaction), and it exhibits a standard deviation of $<0.9\%$ for this packing. It should

also be mentioned that the retention factor of acenaphthene itself has a standard deviation of 2.7%. However, the reproducibility of the selectivity factors is a much better and more important indicator of the reproducibility of a packing than the reproducibility of the retention factors.

III. SPEED AND RESOLUTION

In this section, we will discuss the impact of column length and particle size on the performance of a separation. On first glance, it is already clear that it is not possible to run very fast separations on a long column packed with very small size particles. Conversely, we can also imagine that the separation capability of a short column packed with rather large particles is limited. In the following, we will attempt to shed some light on column performance capabilities in both isocratic and gradient chromatographies.

A. Column Performance in Isocratic Chromatography

The foundation of the views presented here is a series of publications by Martin, Eon, and Guiochon in the mid-1970s.[40–42] Later publications[2,43] cover the material in a similar fashion as described here.

In isocratic chromatography, the analysis of column performance is very straightforward, and we can visualize what is happening with little difficulty. Let us assume that we have a 15-cm 5-μm column with an internal diameter of 4.6 mm! We typically will operate this column at a flow rate of 1 mL/min. Now, let us take a standard separation, and let us increase the flow rate. The separation is happening faster and the peaks are narrower in time units, but we also will loose some resolution, since the peaks will be getting a bit wider compared to the distance between the peaks, as we increase the flow. If we do not have a tight pair of peaks in our chromatogram, we can still recognize that the peaks are getting wider with higher flow rate, since they are also getting shorter. At some point, we cannot increase the flow rate any further, because we would have reached the pressure limit of the instrument.

What will happen, if we do the same thing with a 25-cm column, or with a 5-cm column, packed with the same size particles (5 μm)? We will start off with a higher plate count on the 25-cm column, but we will also hit the instrument pressure limit at a lower flow rate than with the 15-cm column. Conversely, the 5-cm column will have a much lower plate count, but we will be able to operate it with three times the flow rate as the 15-cm column. Figure 7 puts these thoughts into a graphic format. Plotted is the square root of the plate count (which is proportional to the resolution of the separation) versus the analysis time for these three columns. We call the *y*-axis the "resolving power" of a separation. The

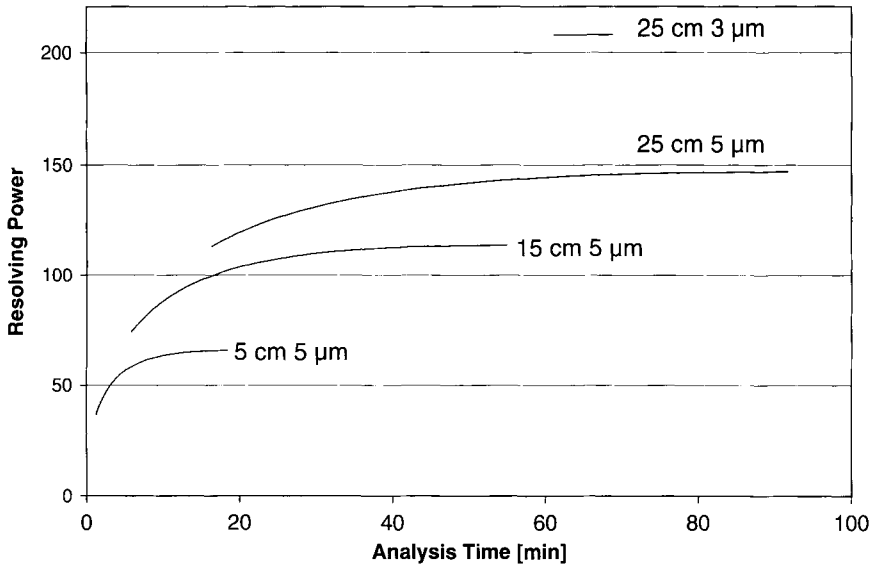


FIGURE 7 **Influence of column length and particle size on the resolving power of a separation.**

x-axis is the analysis time. We can clearly see the pattern of the change in column performance when we change the column length for the three 5-µm columns.

Also included in Figure 7 is the case, when we try to get even more resolving power out of a column by changing the particle size in the 25-cm column to 3 µm. Indeed, we do get what we had wanted, but the analysis time range over which this column can be used is very limited. If we run too slowly, we loose column performance (longitudinal diffusion takes over). We cannot run any faster, since we quickly reach the pressure limit with the 3-µm particles.

The results in Figure 7 are not unexpected. Longer columns have more resolving power, shorter columns provide a faster separation. Reducing the particle size gains resolving power (for a given column length), but it limits the speed of analysis that can be achieved.

There is another way though to think about column performance. This second way will gain speed and often also separation performance at the same time. Let us now change column length and particle size in proportion to each other! Let us compare the performance patterns of a 25-cm 5-µm, a 15-cm 3-µm, and a 10-cm 2-µm column to each other! In this case, the ratio of column length to particle size remains constant. The performance pattern for this set of columns is shown in Figure 8 up to a pressure of 4000 psi (266 bar). Now, each of these columns has the same maximum resolving power. However, as we decrease the particle size, this maximum

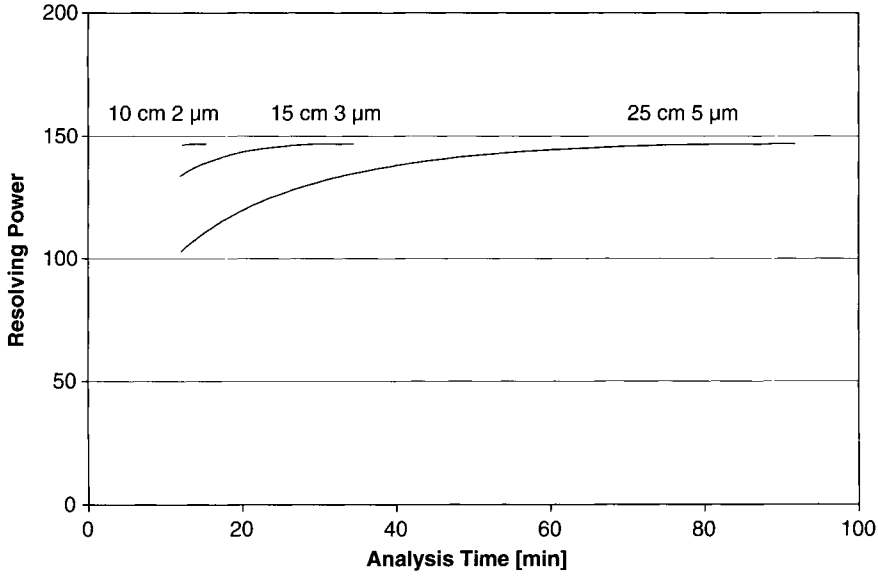


FIGURE 8 **Resolving power as a function of analysis time for various columns with a constant ratio of column length to particle size.**

resolving power is reached in a shorter analysis time. At the point of maximum column performance, the 3-µm column is about 2.8 times faster than the 5-µm column. In addition, we see that all three columns have the same limiting short analysis time. Given the pressure limit, the 10-cm 2-µm column cannot be operated any faster than the 25-cm 5-µm column, but it reaches the maximum column performance at a very short run time.

We have seen now that the maximum column performance remains constant when we change the column length and the particle size in the same proportion. We also have seen that the fastest analysis that can be performed on columns scaled this way remains constant. The advantage of the shorter column with the smaller particles is only that the resolving power at or close to the speed limit is higher for the smaller particles.

How do we get then to a faster analysis? We have to reduce the ratio of column length to particle size. Alternatively, if we want to maintain maximum column performance, we need to go to an instrument that can provide a higher pressure. We cover the latter aspects in Section III.C.

Let us see what happens when we choose another ratio of column length to particle size! In Figure 9, we examine a 5-cm 5-µm, a 3-cm 3-µm, and a 2-cm 2-µm column. Once again we see that all three columns have the same maximum plate count, all three columns can achieve the same shortest analysis time. The maximum resolving power is a little bit less than one half of what was achievable with the five times longer columns shown in Figure 8. But the speed is much higher: the fastest analysis at a

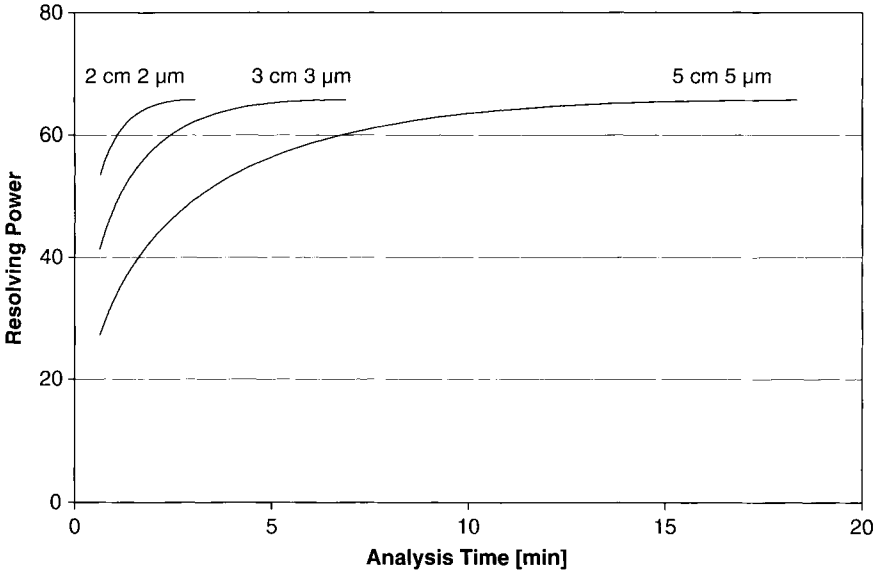


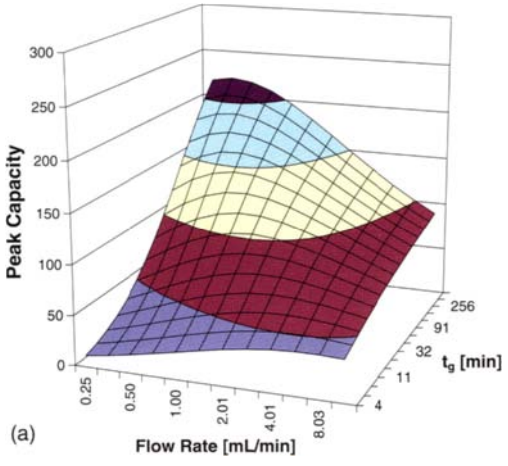
FIGURE 9 **Resolving power as a function of analysis time for various short columns with a constant ratio of column length to particle size.**

pressure of 3000 psi (200 bar) is just around 40 s for our standard analysis to a retention factor of 10. Of course, the smaller particle sizes always outperform the larger particles. Quite reasonable analyses can be performed in less than 2 min with either the 3-µm or the 2-µm columns. Obviously, the focus of the use of columns with a small ratio of column length to particle size is the speed of the analysis, not the maximum separation power.

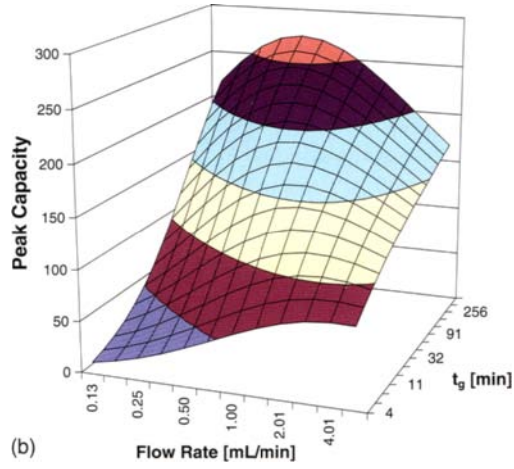
Before we conclude this section, it should be mentioned that the exact speed values that can be achieved depend on the viscosity of the mobile phase. In our examples, we assumed a viscosity of 1 cP, the value for water. If one is using a methanol–water mobile phase, the speed will be a bit slower, whereas for acetonitrile-based mobile phases, it will be a bit higher. However, in these comparisons it was more important to understand the influence of the column parameters such as column length and particle size.

B. Column Performance in Gradient Chromatography

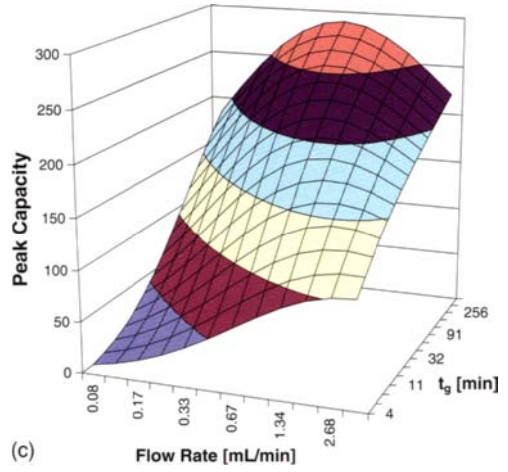
The same principles that we have just applied to isocratic chromatography also apply to gradients. Despite the fact that the use of gradients complicates the picture a bit, the overall rules are similar to what we have seen in isocratic chromatography. We should use a fixed ratio of column length to particle size for the column comparisons in the same


Peak Capacity
300
250
200
150
100
50
0
0.25
0.50
1.00
2.01
4.01
8.03
Flow Rate [mL/min]
256
91
32
11
4
t_g [min]

(a)


Peak Capacity
300
250
200
150
100
50
0
0.13
0.25
0.50
1.00
2.01
4.01
Flow Rate [mL/min]
256
91
32
11
4
t_g [min]

(b)


Peak Capacity
300
250
200
150
100
50
0
0.08
0.17
0.33
0.67
1.34
2.68
Flow Rate [mL/min]
256
91
32
11
4
t_g [min]

(c)

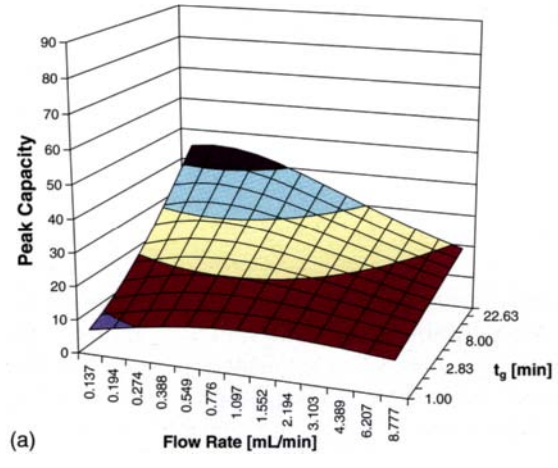
way as we have done in isocratic chromatography. We will assume a fixed gradient from 0% to 100% organic for all the comparisons. In order to measure the separation performance of the gradient, we use the peak capacity, defined as the gradient run time t_g divided by the average peak width w in the gradient:

$$P_c = 1 + \frac{t_g}{w} \tag{3}$$

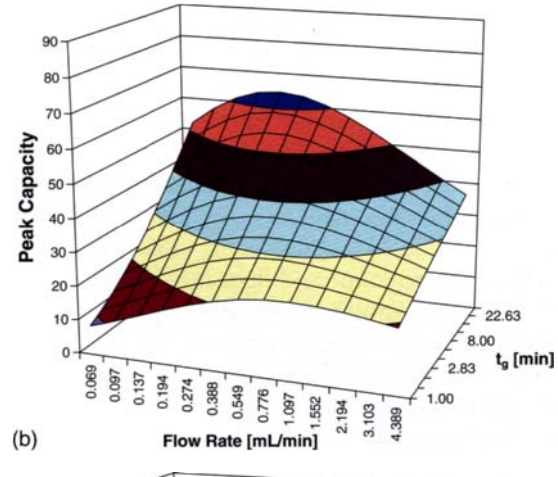
This is simply a measure of how many peaks could be eluted with a nearly baseline separation in a given gradient run time. Other measures for judging the performance of a gradient can be used as well (e.g. reference 44), but the overall findings remain essentially unchanged.

In Figure 10, we have plotted the dependence of the peak capacity on the operational parameters flow rate and gradient run time for three different columns. Before discussing the details of the graphs, let us examine first the nature of the plot. Note that both the flow rate axis and the axis of the gradient duration are logarithmic. This permits a display of the column performance over a wide range of operating conditions. First, let us look at the conditions of a very long gradient run time, which was 512 min (=approximately 8 h) at the back end of the graphs. Under these circumstances, the peak capacity exhibits a maximum. The location of the maximum is at a slow flow rate, close to the optimum of the relationship between plate count and flow rate in isocratic chromatography. As we shorten the gradient run time, the maximum of the peak capacity moves to higher velocities. It also becomes lower. Generally, we obtain maximum resolution, and therefore the best peak capacity, at slow flow rates. However, an analysis time of 8 h or so is not practical. Most gradient analyses are carried out with run times less than 1 h, often even much faster than this. A 30-min analysis is pretty much in the center of the graphs. The flow rate at which the maximum of the peak capacity occurs for this gradient run time is around 1 mL/min for a column with an internal diameter of 3.9 mm. This is in good agreement with the standard operating practices in many labs: gradient run time about 30 min, flow rate about 1 mL/min. The graphs stop on the right at the flow rate where we reach the pressure limit of the instrumentation. In our calculations, this limit was assumed to be around 6000 psi (400 bar).

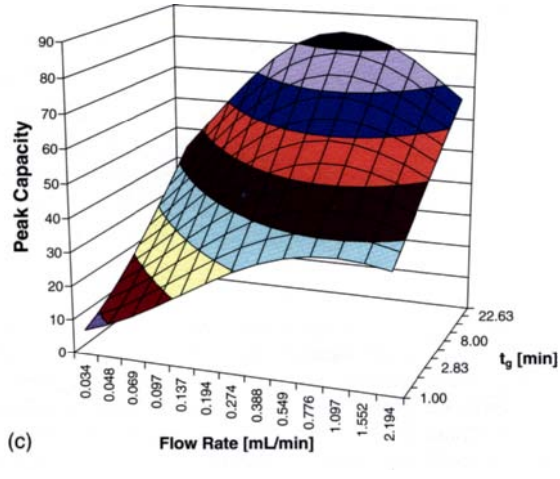
FIGURE 10 **Peak capacity as a function of the flow rate and the gradient duration for three columns with an internal diameter of 3.9 mm. The logarithmic axis for the gradient duration t_g is same for all three graphs and ranges from 4 to 512 min. The logarithmic axis for the flow rate varies from graph to graph. (a) Column: 300 mm × 3.9 mm, 10 μm; flow rate from 0.25 to 11.35 mL/min. (b) Column: 150 mm × 3.9 mm, 5 μm; flow rate from 0.125 to 5.67 mL/min. (c) Column: 100 mm × 3.9 mm, 3.5 μm; flow rate from 0.08 to 3.78 mL/min.**


Peak Capacity
90
80
70
60
50
40
30
20
10
0
0.137
0.194
0.274
0.388
0.549
0.776
1.097
1.552
2.194
3.103
4.389
6.207
8.777
Flow Rate [mL/min]
22.63
8.00
2.83
1.00
t_g [min]

(a)


Peak Capacity
90
80
70
60
50
40
30
20
10
0
0.069
0.097
0.137
0.194
0.274
0.388
0.549
0.776
1.097
1.552
2.194
3.103
4.389
Flow Rate [mL/min]
22.63
8.00
2.83
1.00
t_g [min]

(b)


Peak Capacity
90
80
70
60
50
40
30
20
10
0
0.034
0.048
0.069
0.097
0.137
0.194
0.274
0.388
0.549
0.776
1.097
1.552
2.194
Flow Rate [mL/min]
22.63
8.00
2.83
1.00
t_g [min]

(c)

Now that we have oriented ourselves in the graphs, let us examine them in more detail. In all three graphs, the maximum of the peak capacity at a particular gradient run time moves to a higher flow rate as the gradient run time becomes shorter. The reason for this observation is simple. If we reduce the gradient run time at a particular flow rate, the gradient volume becomes smaller, and we lose resolution. If we want to compensate for this, we need to increase the flow rate. The increase in flow rate brings with it a reduction in column plate count. Therefore, a reduction in run time will always be accompanied by a reduction in peak capacity, but it is not that bad if we increase the flow rate at the same time.

Let us now compare the three graphs in Figure 10 to each other! Figure 10a shows the gradient performance of a 30-cm 10-μm column. Figure 10b represents a 15-cm 5-μm column, and Figure 10c a 10-cm 3.5-μm column. We see that the performance at very long run times improves substantially from the 10-μm column to the 5-μm column, but the increase in peak capacity at the longest run time is only small going from the 5-μm column to the 3-μm column. This is in agreement with the experience shown above for isocratic chromatography: there is a gain in speed, when we reduce the column length together with the particle size, but for the longer run times, the gain in performance is small. However, when we look at the front of the graph, at quick gradient times as fast as 4 min, the advantage of the shorter column packed with smaller particles is substantial (about 30% improvement in peak capacity). At the same time, we are operating close to the selected pressure limit. However, if we compare the gain in speed for equal performance, we can obtain the same peak capacity with the 3.5-μm column in about 4 min as was possible with the 10-μm column in about 30 min.

The smaller particles provide a gain in speed, even in gradient separations. This gain is more pronounced, if we select shorter columns. This is shown in Figure 11, where we compare the peak capacities for 2.1-mm columns of a shorter length. Here we compare 10-cm 10-μm, 5-cm 5-μm, and 2.5-cm 2.5-μm columns. The fastest analysis time considered is a 1-min analysis. The 10-μm column only delivers a peak capacity of around 13, completely inadequate for a real analysis. The 5-μm column reaches a peak capacity of 22. Only the 2.5-μm column reaches an adequate peak capacity, i.e. 37, for this fast analysis. The flow rate is also fairly high for such a short column: 1 mL/min, in agreement with data for fast gradients reported in the literature.[45,46]

FIGURE 11 **Peak capacity as a function of the flow rate and the gradient duration for three fast columns with an internal diameter of 2.1 mm. The logarithmic axis for the gradient duration t_g is same for all three graphs and ranges from 1 to 32 min. The logarithmic axis for the flow rate varies from graph to graph. (a) Column: 100 mm × 2.1 mm, 10 μm; flow rate from 0.14 to 8.78 mL/min. (b) Column: 50 mm × 2.1 mm, 5 μm; flow rate from 0.07 to 4.39 mL/min. (c) Column: 25 mm × 2.1 mm, 2.5 μm; flow rate from 0.03 to 2.19 mL/min.**

C. The Need for Pressure

It is clear that smaller particles are of advantage if one wants to achieve a reasonably high performance at very short analyses times. At the same time, the pressure required to operate a column increases drastically. At a given flow rate, the pressure grows inversely to the square of the particle size. However, the flow rate needed to reach the optimum performance of columns packed with small particles also increases, as the particle size decreases. The consequence of both facts is that the pressure to operate a column under the best conditions increases with the inverse of the third power of the particle size. If we had been comfortable at 750 psi (50 bar) with 3.5-μm particles, we will need 6000 psi (400 bar) to operate the same column packed with 1.7-μm particles at the optimum flow. On the other hand, the gain in speed and performance is very much worth the effort. An example of an isocratic separation that takes advantage of the smaller particles at a constant column length is shown in Figure 12. The measured gain in resolution was 1.5-fold, while the separation was 2.6 times faster. The consequence was

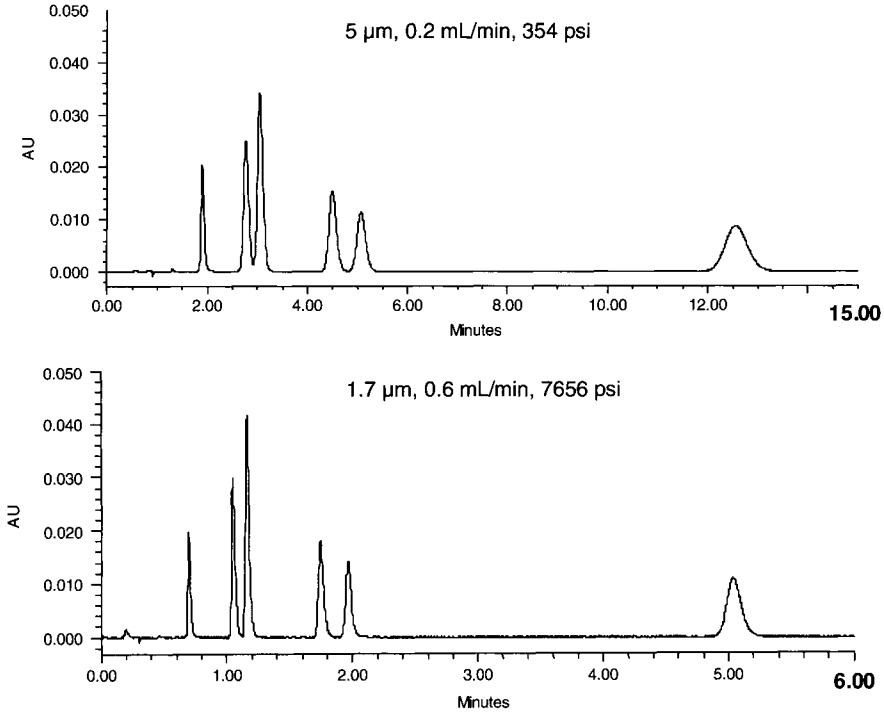


FIGURE 12 **Improvement in a separation by reduction of the particle size at a constant column length of 5 cm. Columns: 50 mm × 2.1 mm. Top: 5 μm. Bottom: 1.7 μm. Chromatogram courtesy of Eric S. Grumbach, Waters Corporation.**

a roughly 22-fold increase in pressure. All of these figures are in close agreement with the theoretical expectations. There is no question of the gain in performance from the very small particles. There is also no question that the cost of this improvement is pressure.

Similar analyses can also be made if the column length is reduced with the particle size, as we have done above. We have shown there that under these circumstances, the performance optimum changes to shorter and shorter run times, as the particle size is reduced. If we continue to do this, we will at some point reach this performance maximum at the pressure limit of the instrument. The only way to further continue on this path towards increased performance is to increase the available pressure.

Small particles are also of advantage for separations that require a very large horsepower in a reasonable time. The first examples of the use of very high pressure by Jorgenson and co-workers focused on maximizing column plate count.[47] In a later publication, the benefit of very small particles was demonstrated also for gradient applications.[48] In more recent times, the instrumentation to execute separations that require a higher pressure and the columns needed for high-power separations have become commercially available.[49] At the time of this writing, a typical particle size for ultra-performance LC applications is 1.7 μm (as in Figure 12) with column lengths up to 15 cm.

An example of an analysis that requires a very high separation power is metabolite profiling in biofluids, i.e. the science of metabonomics.[50] Also, impurity profiling does benefit from the improvement in separation power. Finally, further reduction of interferences in the LC/MS analysis of drugs in biofluids is possible by increasing the peak capacity of the chromatographic separation. The improved separation power results in fewer coelutions for each peak. Fewer interferences mean less ion suppression, which was the surprising side benefit of the performance increase due to small particles when complex separations were performed with MS detection.

IV. SPECIALTY COLUMNS FROM NANOBORE TO PREPARATIVE CHROMATOGRAPHY

In this section, we will briefly touch the subject of columns with both a wider and a smaller internal diameter than commonly used in HPLC. The technology of column preparation has not stood still at these fringes of standard column technology. However, special equipment is required to use these columns. For the proper use of preparative columns, pumps with higher flow rates and injectors with a larger injection volume are required. For the use of columns with a very small diameter, the main technological difficulty is the design of sensitive detectors with very small extra-column band spreading.

A. Preparative Columns

Preparative chromatography can be carried out at many different levels. The preparation of milligram quantities of purified substances from crude synthesis product has become commonplace in the pharmaceutical industry. Often, open-access systems are available that allow an automated purification of the crude product with pre-established chromatographic methods.

The packings used in an open-access preparative system are identical to the ones used for chromatographic analysis. The commonly used particle size is 5 μm, and the common column length is 50 mm, but longer lengths are used as well. Common column diameters range from 7.8 to 30 mm.

The loadability of a column can be estimated by the rule of thumb that column overload will deteriorate a separation if more than 1 mg of sample per 1 g of packing is injected. Another good rule of thumb is that a column contains about 0.75 g of packing per milliliter of column volume. Table 1 gives an estimate of how much sample can be injected on columns of various dimensions before significant overload occurs. Of course, these values need to be taken as guidelines, since for example an overload of the mobile phase buffer or the influence of the sample solvent can result in peak distortion at much smaller injections. A special injection technique, at-column dilution, can be employed to get around early peak distortion due to the sample solvent.[51] In this technique, the sample is injected onto the column in a solvent stream that is compatible with the sample, and this solvent stream is converted just in front of the column to the mobile phase composition required to get good retention and loadability in the preparative chromatography.

TABLE 1 Proposed Operating Conditions and Load for Various Column Dimensions

I.d. (cm)	L (cm)	Flow rate (mL/min)	Load (g)
0.46	5	0.5	0.5–1
0.46	15	2	0.5–1
0.78	5	2	1.5–3
1	5	3	2.5–5
1	10	6	2.5–5
1	15	9	2.5–5
2	5	12	10–20
2	10	25	10–20
2	15	35	10–20
3	5	25	25–45
3	10	55	25–45
3	15	80	25–45

Most preparative columns have some special hardware to stabilize the packed bed. Common techniques used for this purpose are various forms of axial or radial compression. Column stability is improved by increasing the packed bed density. Columns of this type have demonstrably a more predictable lifetime than columns without such features.

B. From Microbore to Nanobore

Columns with diameters of about 4 mm continue to be the most commonly used columns in HPLC. Due to the high linear velocities needed for fast analysis, 2.1-mm columns are often used for very rapid applications. Recently, 2.1- and 1-mm columns have been used for analyses requiring a higher pressure, since the heat generated from the pressure can be dissipated better with a smaller column diameter. Smaller volume columns also generate a higher detector response for equal sample amounts injected, which is of advantage in the analysis of small sample quantities. Thus, there are advantages in the use of smaller diameter columns.

There could be several reasons for the use of columns with a smaller internal diameter. Some MS detectors work best at flow rates lower than standard HPLC flow rates. Fast separations need to be carried out at a high linear velocity. This is best achieved by using a smaller column diameter. In this case, the diameter is best chosen to be around 2 mm. Solvent consumption in a high-throughput lab can be of concern. Under these circumstances, the use of a 3-mm column is often the best solution, since the standard HPLC equipment can be used with little modification. A modern low-dispersion system with a small gradient delay volume is needed for the successful implementation of 3-mm columns.

Another important reason for the use of smaller diameter columns is a limit in the available sample volume. However, as the column diameter is reduced, the peak volumes become smaller as well. The smaller peak volumes require a smaller detector volume (or better, a lower post-column band spreading). If this is not done, the extra-column band spreading can deteriorate the separation. At the same time, a smaller detector volume often goes along with a reduced sensitivity of the detector. Thus, one needs to carefully think which column volumes go along with which detector volumes to not deteriorate the separation power of the columns by too much or compromise detector sensitivity by too much. Part of this thought process should also be the consideration of a sample enrichment on the column by dissolving the sample in a solvent composition that is a weaker eluent than the mobile phase. This permits a larger injection volume, and thus a larger sensitivity, i.e. better limits of detection and quantitation.

There is a reasonable rule of thumb (for isocratic chromatography) that helps in the choice of the right column for the right detector or vice versa: the standard deviation of the system band spreading should not

exceed 1% of the retention volume of the first peak of interest in the chromatogram. For a 15 cm × 4.6 mm column with 10,000 plates, this means that the system band spreading should not exceed 16 μL if the unretained peak is important. Conversely, if only retained peaks are important, a 3-mm column with the same performance can be run on the same system. For a column with an internal diameter of 100 μm, a system band spreading not exceeding 25 nL is desirable. However, these are rough guidelines that should be checked against the performance of the actual column/detector combination.

V. SUMMARY

In this chapter, we have described HPLC columns and packings. The first part deals with the chemistry of the packings, covering both the surface chemistry as well as the solid support matrix. This is followed by a discussion of the physical properties of the columns, i.e. column dimensions and particle sizes. Finally, a section has been devoted to unusual column dimensions. In all cases, special attention has been paid to items that we feel are important for HPLC users in the pharmaceutical industry.

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COLUMN CHARACTERIZATION AND SELECTION

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ABSTRACT

Reversed-phase high performance liquid chromatography (RP-HPLC) methods are used extensively during quality control of pharmaceutical products. During HPLC method development one of the biggest challenges is to select a stationary phase that has the desired selectivity, suitable, reproducibility and stability. Column selection presents difficulties since more than 600 RP-LC column brands are available on the market.

Selectivity of the column and peak shapes of injected compounds is highly dependent on the characteristics of the column. Good understanding of the most significant features of the stationary phases facilitates fast solution of chromatographic issues that occur during method development (e.g. bad peak shape or poor resolution). Properties such as column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric

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selectivity and the amount of metal impurities can be characterized by rather simple chromatographic tests. Calculating representative parameters of the tests allows one to classify stationary phases with similar or dissimilar characteristics. Consideration of classes of chromatographic phases can be beneficial if a column has to be replaced with a similar selectivity column or with an orthogonal stationary phase. Such chromatographic test procedures can also be used to control the performance of a column at any time of its life cycle.

This chapter focuses on reviewing the most important characteristics of RP-HPLC columns that play important roles in real separations. These characteristics can be determined with different chromatographic test methods, which are described in the literature and also summarized here. The results of the test methods provide excellent basis for classification of RP-HPLC columns according to their performance. This type of classification and the existing databases offer great practical assistance in selection of appropriate RP-HPLC columns. One of the databases is freely accessible through the Internet.

I. INTRODUCTION

Today, the separation mode of choice for the majority of high-performance liquid chromatography (HPLC) analyses is reversed-phase liquid chromatography (RP-LC). Chromatographers within the industrial settings mostly use RP-LC systems of conventional size, i.e. columns with internal diameters of 4–5 mm and 10–25 cm length. Many HPLC methods are described in the official compendia (e.g. European Pharmacopoiea[1] (Ph. Eur.), United States Pharmacopoeia[2] (USP)) and almost all use RP columns. As is the case today, most of the columns for RP-LC separations are manufactured from silica substrates. Silica has many favorable properties, making this material nearly ideal as a support for RP columns. The broad range of compounds that can be analyzed (e.g. non-ionic, ionizable and ionic compounds) has caused this widespread applicability. Other main contributions are the short equilibrium times and the possibility to perform gradient analysis.[3] The opportunity to use water-rich eluents and samples during RP-LC separations is also beneficial from an environmental point of view.

Today, an overwhelming number of RP columns are available on the market. Although they belong to the same type of column category, they differ in type of ligand, end-capping, type of silica, residual silanols, bonding density, pore size, etc.[4] Several papers have been published that show the great differences in chromatographic properties between different brands of RP-LC columns.[5–8] Consequently, the selectivity of stationary phases of various manufacturers is considerably diverse.

Since more than 600 different RP-LC columns are available, it is extremely difficult to select the optimal column for a given application. One has to find a stationary phase that retains the compound(s) of

interest with a reasonable retention factor. The column has to have certain selectivity in order to obtain good separation of the analytes and peak shapes of the compounds have to be acceptable.

It is often the case that the stationary phase, which is optimal for a certain separation, cannot be used any longer. For example, sometimes the ideal column is no longer available on the market or difficult to purchase. The lot-to-lot reproducibility of the chromatographic support is occasionally not sufficient enough to transfer a method from one laboratory to another. In these and other cases, the analyst has to find a replacement stationary phase that has very similar characteristics to the original one.[9]

The pharmaceutical industry often employs more than one chromatographic conditions with different selectivities for impurity profiling of drugs. Applying in parallel two or more methods maximizes the possibility that all substances can be unveiled.[10] The type of stationary phase has one of the most important influences on the orthogonality of chromatographic systems.[11] In this case, the goal is to select few supports with significantly different selectivity. Efficient column selection is essential in order to decrease the orthogonal method development time.

Manufacturers provide only limited information about the stationary phases, e.g. the results of their own test method and some applications. Since manufacturers use different tests and evaluation parameters for their columns, comparison of stationary phases from different manufacturers is difficult.[12] Due to these facts the choice of a proper RP-LC column for a particular separation, for finding a replacement or for orthogonal support is demanding.

Official compendia also do not mention the brand of the stationary phase(s) that can (has to) be used in order to obtain sufficient selectivity during a given RP-LC analysis. Thus monographs do not give precise information about column identity that would allow obtaining correct and reproducible results. In the description of a liquid chromatographic (LC) method, the Ph. Eur.,[1] the USP[2] and other compendia only give the precise eluent composition and other conditions (e.g. temperature, gradient parameters, etc.). Instead of mentioning the brand name of the stationary phase, which is not allowed to be communicated in the official monograph, the Ph. Eur. prescribes a system suitability test (SST) and further refers to a description of the stationary phase in the reagents part with particle size, pore size, specific surface area and chain length. The USP has divided the stationary phases into 61 different groups (L1–L61). It provides detailed information about the column chemistry, particle shape and diameter. Column manufacturers or sources and materials stated in USP monographs are only recommendations. This information is often insufficient to choose a suitable column.

Engelhardt et al. performed the determination of impurities of salicylic acid according to the Ph. Eur. on three different commercially available RP columns.[13] With one of the columns, all acidic solutes co-eluted with the solvent; in the other columns changes in the elution sequence were observed. This example demonstrates the problem that may occur if the column properties are not sufficiently described. Steffeck et al. also draw attention to the difficulties related to RP-LC column selection.[14]

Recently, faster method development procedures have used aggressive eluents under elevated temperature conditions in order to improve selectivity, efficiency and resolution, reduce solvent consumption and also decrease analysis time.[15] Since chemical and thermal stability of silica-based phases is limited, the chromatographic characteristics of stationary phases change during usage. Therefore, the selectivities of the columns are not constant. The stability of Zorbax Extend® column is demonstrated in Figure 1.[16] If a method is developed on a brand new column, it is not guaranteed that a used stationary phase from the same brand would give the same separation and vice versa.

The nomenclature of RP-LC stationary phases is not clear and therefore confusing. Unified characterization and classification of columns into groups with similar characteristics would make the column selection easier. Unfortunately, there is no such characterization and classification procedure that is widely accepted.

From the beginning of the development of RP-LC phases, extensive research has been done on evaluation methods for characterization of RP-LC stationary phases, resulting in a substantial number of books and papers on this issue.[6–8,17–72] However, chromatographers get confused which test method to select to characterize columns and there is no universally accepted test procedure.

Various research groups have been improving the existing testing methods and developing new methods for testing RP-LC phases.[18–20] They generally run chromatographic test methods on different stationary phases to investigate physicochemical interactions between simple well-characterized analytes and a stationary phase. Column properties, e.g. efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the amount of metal impurities, are usually evaluated from the test separations. These chromatographic parameters, which are calculated for different stationary phases, offer a possibility to compare columns on a quantitative basis. Since several parameters are usually employed to characterize one column and authors study numerous columns at the same time, large databases are established.

Statistical methods facilitate the interpretation of these databases. In the literature, the chromatographic approaches have been combined

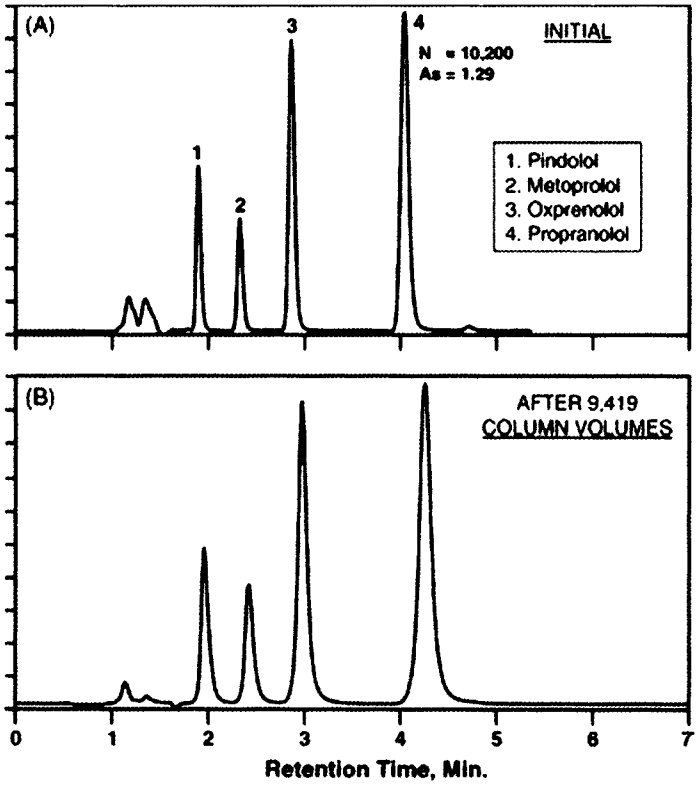

FIGURE 1 **(A) Initial. (B) After 9.419 column volumes. Stability of bidentate C18 column at pH 11. Column: Extend® C18 column 15 cm × 0.46 cm (Agilent Technologies, Newport, DE, USA). Sample: β-blockers; eluent: acetonitrile–0.017 M aqueous potassium phosphate buffer, pH 11.0 (50:50, v/v); flow rate: 1.5 mL/min; temperature: 23°C. (Reprinted with permission of the publisher from reference 16.)**

with different chemometric tools such as principal component analysis (PCA), cluster analysis and radar plots. These tools further visualize groupings in order to characterize RP packing materials and to try to gain a better understanding of the underlying molecular interactions between the analyte and the stationary phase material.[6,8,21,22] Especially, PCA has been shown to be extremely useful in simplifying the "data mining" process for large amounts of data. PCA provides a simple graphical comparison of the phases within the database. The stationary phases, which appear close to each other on the PCA plot, have similar characteristics. The greater the distance between two columns on the PCA plot, the more different they are. It has been also verified that columns having closely related characteristics as determined by these chromatographic tests are suitable for the same chromatographic application.[23]

This chapter focuses on reviewing the most important characteristics of RP-HPLC columns, which play an important role in real separations. These characteristics can be determined with different chromatographic test methods, which are described in the literature and will be summarized here. Commercially available test mixtures and their usage will also be discussed. The parameters, which are evaluated form the results of the test methods provide an excellent basis to classify RP-HPLC columns according to their performance. Classification and the existing databases, which will also be reviewed, offer great practical assistance in selection of appropriate RP-HPLC columns.

II. CHARACTERISTICS OF RP-HPLC COLUMNS AND CHROMATOGRAPHIC TESTS

A number of factors influence the properties of silica-based RPs. The nature of the silica is characterized by the particle diameter, specific surface area, pore diameter, pore volume, chemical purity and acidity. The silane bonding (e.g. length of the alkyl group, the usage of mono-, di- or trichlorosilanes, the surface concentration of bonded alkyl groups and the amount of unreacted, accessible silanol groups) also affects the properties of the RP stationary phases.[24]

Properties of RP-LC columns can be characterized by non-chromatographic, spectroscopic and chromatographic methods. Carbon content, amount of metal impurities, particle size, particle shape, specific surface area, pore size, porosity, packing density and acidity can be determined by non-chromatographic methods. However, these techniques are not easy to perform and cannot be carried out on the packed column without destruction. It has also been proven that the physical parameters (e.g. carbon load, particle size and surface area) often show little correlation with the performance of the column.[25]

Spectroscopic characterization of stationary phases is usually performed using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. Bonded phase, silanols and silanes on the solid support can be identified and information about the type of bonding can be qualitatively obtained using diffuse reflectance infrared Fourier transform IR.[26] The various types of silanols (isolated, geminal and vicinal) can be determined using ^{29}Si solid state NMR, whereas the type of bonding (mono, di or trifunctional) and type of end-capping can be determined using ^{13}C solid state NMR.[27] A disadvantage of spectroscopic techniques, however, is that they can be used only to determine bulk characteristics of the stationary phase. Spectroscopic techniques (e.g. ^{29}Si NMR) deliver complex data of surface characteristics for which the relation to chromatographic observations is uncertain.[24]

The chromatographic approaches measure physicochemical interactions between simple well-characterized analytes and a stationary phase.[28]

Since subtle differences between the phases may have a large impact on the shape of the chromatographic peak, a chromatographic characterization is preferred. Another advantage of the chromatographic characterization is that the stationary phases can be tested under the same condition as they are used in daily practice, such as in packed columns.[3]

The chromatographic characterization of stationary phases can be divided into three classes:[3]

1. Empirical-based evaluation methods: this section can be subdivided into two groups – (i) evaluation based on chromatographic data of test compounds chosen according to a certain line of thought, and (ii) evaluation based on chromatographic data of dedicated test compounds. In the case of testing columns for the applicability in analyzing basic compounds, basic compounds are used as test probes.
2. Thermodynamically based methods: the obtained information is based on studying enthalpies and entropies of transfer of solutes from the mobile to the stationary phase (e.g. reference 29).
3. Evaluation methods based on a retention model: the obtained information is based on a specific retention model such as quantitative structure retention relationship (QSRR) studies (e.g. reference 30).

In this chapter, various empirical-based methods for characterizing columns are discussed. These simple chromatographic tests are very easy to apply. They can be used as an everyday practice in any laboratory. The results of these chromatographic tests provide practical information about a given column and provide useful basis for column classification. Properties such as column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the amount of metal impurities can be characterized by these chromatographic tests.

Forlay et al. measured theoretical plate numbers and symmetry factor values for three solutes in various stationary phases and different mobile phase compositions with the aim of grouping the systems and studying the possibilities for replacement of columns and eluent compositions.[31] They concluded that the monofunctional test compounds could only be used with caution for selection of columns for high molecular mass, multifunctional solutes.

A. Column Efficiency

Column efficiency is the degree to which species flow through the column as "bands," without being spread; less band broadening implies a less likely overlap of peaks in the chromatogram. The efficiency terms are derived from the treatment of the chromatographic column as being

made up of a number of discrete narrow bands called theoretical plates, similar to a distillation column. The efficiency gives information about the quality of the filling process and the physical properties of the particles. The efficiency mostly depends on the particle size and on the alkyl-chain density on the silica surface.[8,32] Efficiency is generally characterized in terms of theoretical plate number, theoretical plate height or reduced theoretical plate height.

Column efficiency is usually measured using non-polar compounds, especially aromatic hydrocarbons. McCalley used benzene,[33] Engelhardt employed toluene and ethylbenzene,[34] Tanaka used propylbenzene, butylbenzene and amylbenzene,[35] Neue used acenaphthene,[36] Goldberg used terphenyl and biphenyl;[37] 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) was employed by Daldrup.[38] Methanol–water,[37] acetonitrile–water[6,35,36] or aqueous buffer mixtures[38] are generally used as eluent.

B. Hydrophobicity, Methylene Selectivity

The chemical nature of the stationary phase will govern how strongly the analyte is retained. Hydrophobicity of the column is considered as the retentivity for compounds based on interactions between the compound and the ligand on the silica.

Hydrophobicity is calculated as retention values of aromatic hydrocarbons. Köhler used 1-phenylheptane-1-phenylhexane,[39] Neue employed acenaphthene[35] and Eymann used nitrobenzene, toluene and trifluoromethyl-benzene[40] to calculate the retention factor (k'). Guiochon used the retention times of toluene, ethylbenzene and butylbenzene as a measure of hydrophobicity.[41]

Methylene selectivity is the ability of a given stationary phase to separate structurally closely related compounds.[10] In practice, it reflects the possibility of the phase to separate two molecules, which are different only in one methylene group.

Methylene selectivity is measured by the selectivity factor (α) between non-polar alkyl benzenes in a homologous series, differing by one methylene group. Engelhardt[32,34] and Tanaka[35] used ethylbenzene and toluene, Cruz employed amylbenzene and butylbenzene,[8] Goldberg used anthracene and naphthalene[37] for determination of the methylene selectivity. Use of methanol/water or methanol/aqueous buffer mixtures as mobile phases is typical for these experiments.

Claessens et al. compared five different test methods for RP-LC columns.[12] Good correlation was found between column efficiency, hydrophobicity and methylene selectivity. The results of these different tests are usually interchangeable and column classification by these methods will provide similar patterns.

If the separation between hydrophobic compounds is not satisfactory, the column can be replaced with a more efficient one. In RP-HPLC

higher density columns (e.g. C18 instead of C5-8 or higher carbon load chromatographic supports) and smaller particles result in narrower and better-separated peaks.[10]

C. Silanol Activity

Silanol groups, which remain on the silica surface due to incomplete derivatization, play a key role in the retention mechanism. The silanol activity reflects the influence on the solutes' retention caused by these silanol groups. Since large differences between packing materials exist due to different manufacturing processes used, large differences between peak shapes are often obtained with various stationary phases. Since symmetrical peaks are favorable for high selectivity and sensitivity, determination of silanol activity of a given stationary phase is important in order to select a suitable stationary phase for a specific separation. Compared to hydrophobic properties, determination of the silanol activity of RP stationary phases is a more difficult topic as reviewed by Nawrocki.[24]

It was an early observation by Halasz et al. that a small retention factor of nitrobenzene compared to naphthalene or benzene using *n*-hexane as eluent (normal phase mode) indicates a lack of silanol interactions.[42] Other empirical tests described in the literature are mainly based on relative retention values between compounds from which the retention is assumed to be caused by hydrophobic and silanol interactions, and compounds from which the retention is assumed to be based on hydrophobic interaction only. For example, large selectivity factors for *N*,*N*-diethyltoluamide/anthracene[43] or caffeine/phenol[35] are an indication of free silanol groups. Separation of *ortho*-, *meta*- and *para*-toluidine indicates active silanol sites because their hydrophobic properties are identical but their basicities are different.[34–39]

Later, basic compounds were used to indicate silanol activity in the RP mode. Aniline derivatives,[44] pyridine or its derivatives[7,41] and basic drugs (propranolol, amitryptiline)[36] are applied for these measurements. Poor peak symmetry and/or large retention of basic compounds show the activity and accessibility of free silanols on the silica surface.

From a historical point of view, clear differences in the development of column test methods can be observed. The test methods developed by Gonnet, Daldrup and Walters[5,38,43] were developed from 1982 to 1987. The tests of Tanaka[35] and Engelhardt[13] are more than a decade old, meaning that these tests were developed using stationary phases of the same period. Since that period many new stationary phases have been developed. The differences between these phases with respect to ionic interactions will be small compared to the "older phases." Vervoort believed that the "older" tests are suitable to differentiate between generations of columns.[22] The differences between a Symmetry C8 and a Hypersil MOS column using the test of Engelhardt are clearly shown in

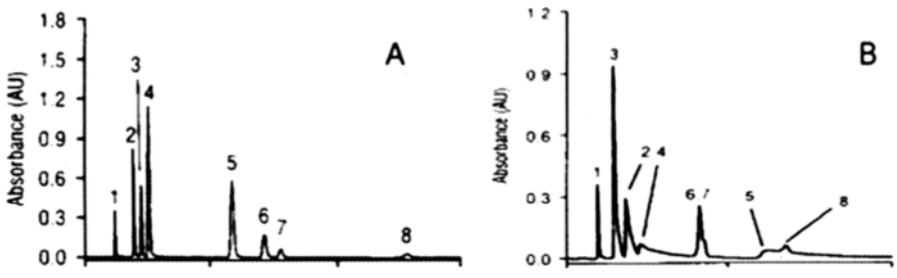

FIGURE 2 Separation of the Engelhardt test mixture using a Symmetry C8 (A) and a Hypersil MOS (B) column. Eluent: methanol/water (49/51% w/w). Analytes: (1) thiourea, (2) aniline, (3) phenol, (4) *m*- and *p*-toluidine, (5) dimethylaniline, (6) benzoic ester ethylester, (7) toluene and (8) ethylbenzene. (Reprinted with permission of the publisher from reference 32.)

Figure 2.[32] Vervoort also concluded that for the modern phases only subtle differences are present, which cannot be visualized using tests like the Engelhardt and Tanaka tests. Characterization of stationary phases for the analysis of basic pharmaceuticals is best performed using basic compounds as test probes.

The large varieties of the tests, which are described in the literature, make it difficult for the user to judge the quality of these tests. Claessens et al. found that different silanol activity test results were generally not in mutual agreement and not interchangeable, so column classification on silanol activity depends on which test method is applied.[12] Buffering of the eluent greatly influences silanol activity test results because it reduces the dissociation of silanol groups. According to Claessens, for the sake of objective column comparison and ranking, buffering of the eluent for such tests is mandatory. However, some authors use buffers, others do not.

To improve the peak shape, optimization of the mobile phase can also be considered. Several approaches are published that discuss how to reduce the ionic interaction between analyte and acidic sites on the column packing.[45]

D. Ion-Exchange Capacity

Compounds with a basic nitrogen atom in the chemical structure often cause problems when analyzed with RP-LC. Asymmetrical peaks, irreproducible retention and non-robust separation methods are frequently obtained. Basic nitrogen atoms can be protonated, depending on the pK of the analyte and the pH of the eluent. These protonated basic compounds can interact with residual silanol groups of the stationary phase, as shown in the equation:

$$XH^+ + SiO^-Na^+ \rightleftharpoons Na^+ + SiO^-XH^+ \quad (1)$$

Thus, besides the RP retention mechanism an ion-exchange retention mechanism occurs and results in distorted peak shapes.[3]

Ion-exchange capacity can be characterized by measuring the difference in selectivity coefficients of a base and a neutral compound at low and relatively high pH values. The difference should be small. In most cases, the selectivities of benzylamine and phenol are compared at pH 2.3 and 7.6.[18,35] Guiochon employed a relative elution parameter of procainamide and benzylamine.[41]

E. Steric Selectivity/Shape Selectivity

The steric selectivity expresses the possibility of a stationary phase to separate two molecules differing in their three-dimensional structure. The factors that affect the chromatographic discrimination of compounds on the basis of molecular structure, namely "shape selectivity," were reviewed in terms of contributions from bonded phase morphology, and in terms of operational conditions. An emphasis was placed on practical choices that are available to control selectivity and optimize separations for isomers and related mixtures.[46]

One of the first studies, which investigated steric selectivity of RP-LC columns, was published by Sander and Wise.[47] They investigated the preparation of series of C_{18} monomeric and polymeric phases as well as an intermediate class of phases ("oligomeric phases"). They characterized the stationary phases' surface coverage, carbon loading and performed empirical LC tests. The latter test consisted of two LC procedures. The separation of a 16-component polycyclic aromatic hydrocarbon mixture (SRM 1647) was performed by using a linear gradient of 40–100% acetonitrile/water. A three-component test mixture (phenanthro[3,4-*c*]phenanthrene (PhPh), benzo[*a*]pyrene (BaP) and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) (dibenzo[*g,p*]chrysene)) was chromatographed isocratically at 85% acetonitrile/water. The separation of the two mixtures on representative monomeric, oligomeric and polymeric phases is shown in Figure 3.

The polymeric phases were found to be most selective toward polycyclic aromatic hydrocarbon (PAH). Selectivity toward PAH increased with increasing surface coverage of the bonded phase. The selectivity of the oligomeric phases toward PAH was intermediate to the selectivity of the monomeric and polymeric phases. Column selectivity is directly related to bonded phase surface coverage values while absolute retention is more closely related to the amount of carbon contained within the column. The effect of end-capping on the separation of the PAH mixture was negligible; however, retention was slightly longer on the non–end-capped phase.

The elution order of a three-component mixture, PhPh, TBN and BaP, was strongly dependent on the type of phase and the surface coverage. Since BaP consists of five condensed aromatic rings and both PhPh and TBN contain six condensed rings, it might be expected that BaP would

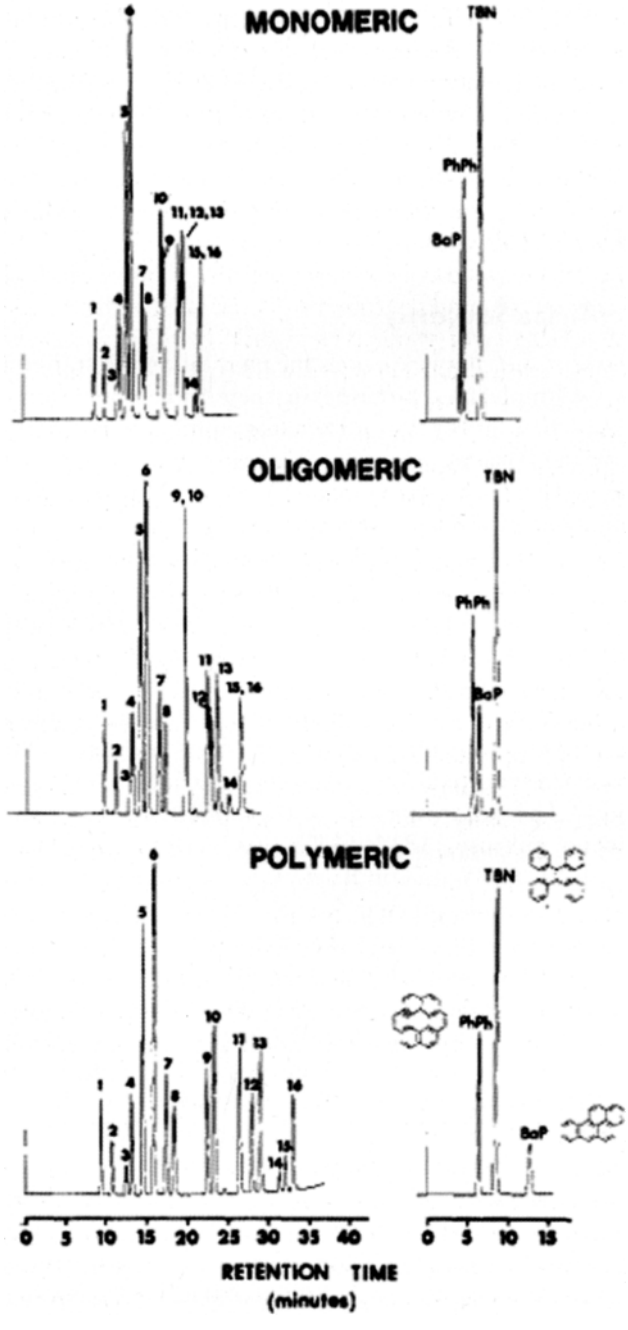

FIGURE 3 Separation of 16 polycyclic aromatic hydrocarbons (SRM 1647) on representative monomeric, oligomeric and polymeric phases. Separation of the 16-component mixture was performed by using gradient elution, 40–100% acetonitrile

elute before PhPh and TBN in all RP systems. However, this elution order was observed only for the monomeric phases. For the oligomeric and polymeric phases the retention of BaP relative to PhPh and TBN increased. The unusual retention behavior of these three compounds is probably related to the shape of the molecules. PhPh and TBN are non-planar; PhPh is helically shaped, while TBN is shaped like a saddle. BaP, however, is completely planar. Apparently, the planar shape of BaP permits an enhanced interaction of this molecule with the polymeric phases.

Shape selectivity can be determined using aromatic hydrocarbons, where one is twisted and the other is planar. Besides the Sander and Wise test,[48] the selectivity of triphenylene (TRI) (planar) and *ortho*-terphenyl (*o*-TER) (twisted) is used as a measure. The mobile phase usually contains methanol/water in this test.[18,35]

Engelhardt et al.[49] compared the use of the TRI/*o*-TER selectivity, α(TRI/*o*-TER), in the Tanaka test with the well-known shape selectivity test of Sander and Wise, based on selectivity measurements between BaP and dibenzo[*g,p*]chrysene.[48] The study shows that both tests correlate well in their ability to distinguish between "monomeric," "intermediate" and "polymeric" phases in terms of shape selectivity. In the study of Engelhardt, it was further concluded that those columns, which have α(TRI/*o*-TER) values larger than 3, show good shape selectivity. Engelhardt also pointed out that besides carbon loading and a certain degree of polycondensation of silanes at the surface, the accessibility of these groups is also of considerable importance to obtain shape selectivity.

Polymeric C18 phases usually have unique steric selectivity, which makes them especially suited for the separation of polycyclic aromatic hydrocarbons and isomers.[46] Visky et al. observed, using the TRI/*o*-TER selectivity test, that most of the highest steric selectivity columns are polar-embedded phases.[19] Euerby and Petersson also found that the polar embedded materials were differentiated from the other phases due to their high shape selectivity character.[18] It can be concluded that the polymeric and the polar embedded C18 are the most suitable supports if a steric factor plays important role in the separation mechanism.

FIGURE 3 Cont.

in water over 30 min at 2 mL/min. The three-component mixture was run isocratically at 85% acetonitrile/water. The elution order of benzo[*a*]pyrene (BaP), phenanthro [3,4-c] phenanthrene (PhPh) and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) is indicative of phase type. Component identification: (1) naphthalene, (2) acenaphthyiene, (3) indeno[1,2,3-*cd*]pyrene, (4) fluorene, (5) phenanthrene, (6) anthracene, (7) fluoranthene, (8) pyrene, (9) benz[*a*]- anthrancene, (10) chrysene, (11) benzo[*b*] fiuoranthene, (12) benzo-[*k*]fluoranthene, (13) benzo[*a*]pyrene, (14) dibenz[*a,h*] anthracene, (15) benzo[*ghi*]perylene, (16) indeno [1,2,3-*cd*]pyrene. (Reprinted with permission of the publisher from reference 47.)

F. Presence of Metal Impurities

Nowadays, manufacturers prepare RP-columns from highly pure silica. However, the chromatographic supports still contain very small amounts of metal contamination. Metal ions can also get into the stationary phase from mobile phases and from the cartridge during usage. Metal impurities can influence the chromatographic properties of stationary phases and thus also the performance of a chromatographic analysis. Metals can increase the acidity of adjacent silanol groups that may then enhance silanol activity. The metal contamination ions may be strong adsorption sites for complexing solutes.

Metal impurity on the silica surface can be examined using chelating agents. To determine the metal content of stationary phases chromatographically, various suggestions have been proposed. The first test solute was proposed by Verzele and Deweale.[50] Peak tailing and retention of acetylacetone was a measure of metal contamination.[20,41] It was demonstrated later that the peak of acetyl acetone was also affected by keto enol tautomerism effects and therefore the effects observed cannot be solely attributed to metal activity.[51]

Peak tailing of 2,2′-dipyridyl[40,41] or 2,3-dihydroxynaphthalene[8,40,41] is observed in the presence of metal contamination. Small theoretical plate numbers, strong retention and poor peak symmetry of these compounds are also indications for metal ions on the silica surface. Measurements have to be carried out at optimum pH where these compounds can form complexes with metal ions. Engelhardt and Lobert described the use of 2,2′-bipyridyl and 4,4′-bipyridyl to determine metal impurities. 2,2′-bipyridyl can form complexes with metals, whereas 4,4′-bipyridyl cannot. Therefore, the relative asymmetry of both compounds was found to be a good measure of metal content. Figure 4 shows examples for various columns.[52]

A comparative study of the different methods of verifying trace metal presence has not been reported. It is unclear whether different tests described in the literature are providing similar information.

G. Commercially Available Test Mixtures

Several manufacturers offer test mixture kits. Sigma-Aldrich markets two test mixtures. Reversed-Phase Mix 1 contains uracil, acetophenone, benzene and toluene. The composition of Reversed-Phase Mix 2 is uracil, *N*,*N*-diethyl-*m*-toluamide, phenol and toluene. The latter is also distributed by Alltech. Cerilliant HPLC Reversed-Phase Testmix consists of five components: methylbenzoate, *p*-nitroaniline, phenetole, theophylline and *o*-xylene. Column efficiency, hydrophobic selectivity and silanol activity can be characterized using these test kits. The reader is referred to the user manuals of these products.

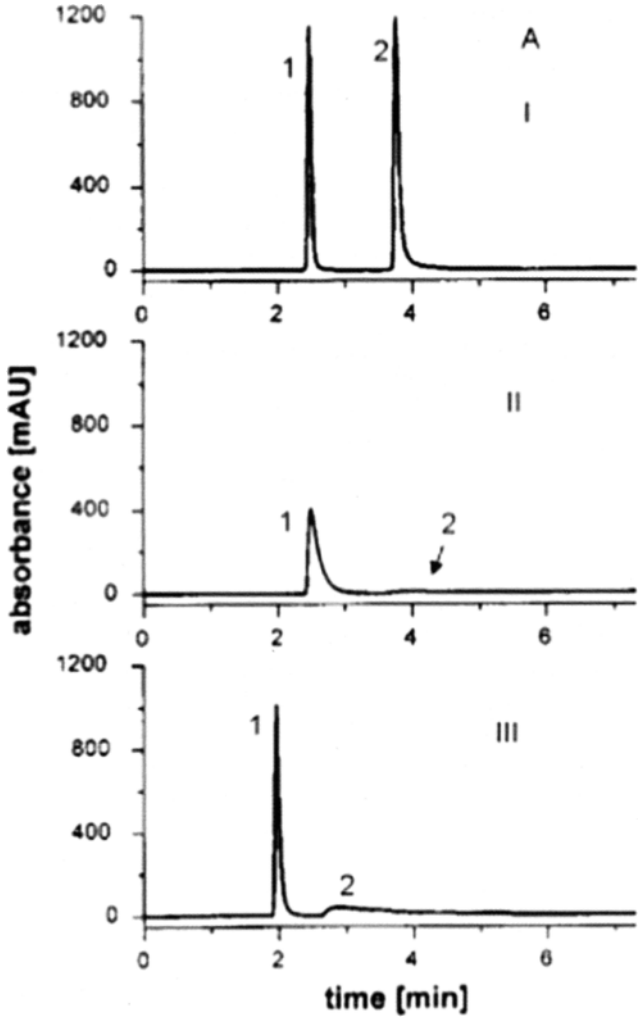

FIGURE 4 Determination of metal activity on RP 18 columns. (A) Differentiation between silanophilic and metallophilic activity; (I) low silanophilic and low metallophilic activity, (II) high silanophilic activity, high metal content, (III) low silanophilic activity and high metal content. The eluent used was methanol/water (49/51% w/w), the flow-rate was 1.0 mL/min, the column temperature was set to 40°C and detection was performed with UV at 254 nm. (Reprinted with permission of the publisher from reference 52.)

Standard Reference Material® 870[53] is a mixture of five organic compounds in methanol intended for use in characterizing general aspects of LC column performance, including efficiency, void volume, methylene selectivity, retentiveness and activity toward chelators and organic bases. Steric selectivity cannot be characterized with this test mixture. SRM 870 consists of a mixture of the following five organic compounds in methanol: uracil, toluene, ethylbenzene, quinizarin and amitriptyline. The recommended composition of the mobile phase is 80% methanol and 20% buffer, where the buffer composition is 20 mmol/L potassium phosphate adjusted to pH 7.0 ± 0.1. Separations of SRM 870 with detection at 254, 210, and 480 nm are shown in Figure 5.

In this test, uracil is employed as an indicator of the void volume (unretained volume). Toluene and/or ethylbenzene are useful markers for calculation of column efficiency (theoretical plates, *N*). The retention of

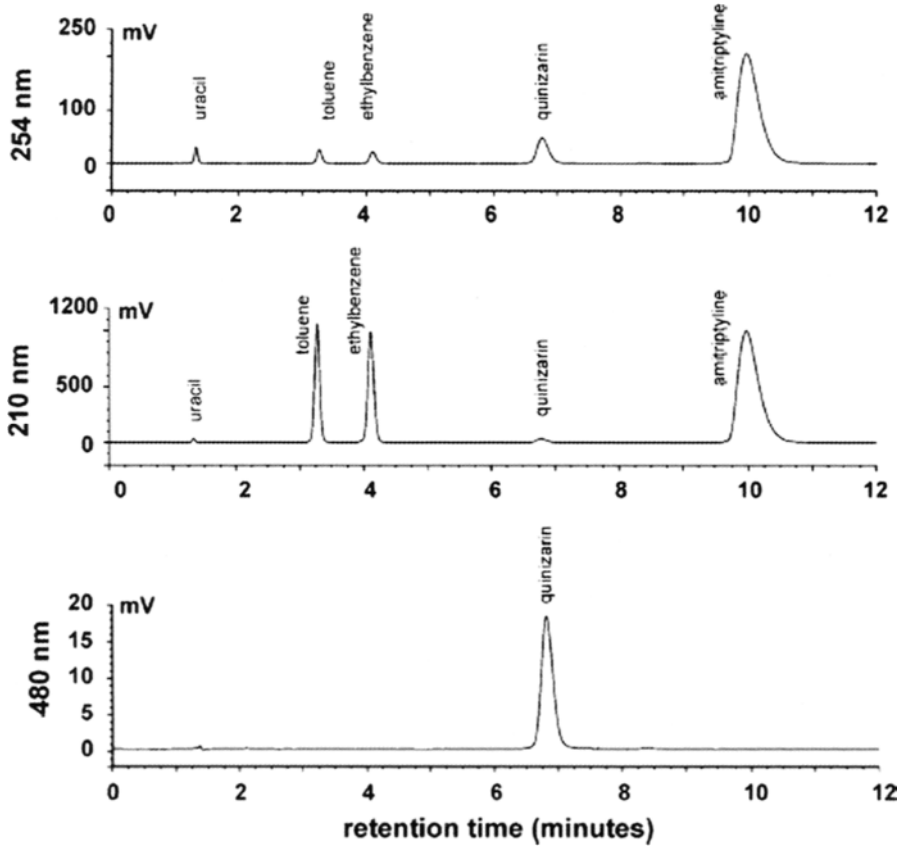

FIGURE 5 Separation of SRM 870 with detection at 254, 210 and 480 nm. (Reprinted with permission of the publisher from reference 53.)

toluene and ethylbenzene can be considered as a measure of hydrophobicity. Methylene selectivity is determined by the selectivity factor of toluene and ethylbenzene. Tailing factor of amitriptyline, which is a basic ($pK_a = 9.4$) pharmaceutical drug, is associated with silanol activity. The retention behavior of quinizarin (1,4-dihydroxyanthraquinone), which is a metal chelating reagent, is expected to be indicative of the presence or absence of metals in the chromatographic system. Low activity toward chelating reagents is indicated by a symmetrical peak shape; high activity toward chelating reagents is indicated by a tailing, asymmetrical peak shape. This test is very accessible and this is the most comprehensive data about the condition of a given column. It can be applied routinely in any laboratory and data evaluation is also very straightforward.

Restek recommends NIST 870 standard and another test mixture. The composition of the latter test mixture is uracil, benzene, naphthalene and biphenyl. Routine analysis using these mixtures can assist in determining the need to perform column and/or system maintenance.

III. COLUMN CLASSIFICATION AND SELECTION

As noted previously, hundreds of different brands of RP-LC columns are available on the market. Therefore the selection of a suitable RP-LC column is very difficult. The need for characterization and classification of RP-LC columns has been rising since the 1970s. Several research groups, even the pharmacopoeias, have been trying to classify RP columns in order to provide assistance in column selection (see Section I).

An interesting methodology, which has been employed by several research groups, is to characterize the stationary phases with different experiments, evaluate certain chromatographic parameters from the experiments and classify them based on those parameters.[18,20,54] As was described in the previous section, the chromatographic characterization approach is the most meaningful because it seeks to measure and specify, discrete physicochemical interactions between certain simple and well-characterized analytes and a stationary phase. Numerous protocols have been published in the literature, which will be reviewed below. The most useful ones allow one to measure a number of parameters that reflect the most important chromatographic characteristics, e.g. column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the amount of metal impurities. The various chromatographic tests were performed on a large number of stationary phases by different research groups. As a result, huge databases were published in the literature, which provide an excellent basis for column classification.

It is usually very difficult to evaluate data from large datasets. Statistical methods are useful in data mining, e.g. to cluster groups of RP-LC phases of similar chromatographic properties. Such methods can

effectively facilitate column classification and selection.[55] PCA is one of the most utilized methods for interpretation of large amount of data. In PCA, the number of variables (e.g. column characterization parameters) is reduced by a projection of the objects (e.g. stationary phases) onto a smaller number of new variables termed principal components (PC). The projection of objects onto a PC is called scores – by plotting the scores for two PCs it is possible to graphically find similarities and differences between objects (stationary phases). The distance between objects in a score plot shows if they are similar or different. How much of each of the original variables that are included in a PC is described by so-called loadings, one for each variable. By plotting the loadings for two PCs, it is possible to see which of the original variables are most important (longest distance from the origin) and if any variables are correlated (the same or opposite directions on a straight line through the origin). The reason why two objects are different can easily be determined with a so-called contribution plot. This type of plot shows which variables (chromatographic parameters) cause a difference between two objects (stationary phases) or, alternatively, one object and the average object.

Chromatographic characterization procedures combined with chemometric evaluation techniques have been used in the literature to classify columns with similar characteristics.[56,57] Walczak et al. investigated the chromatographic behavior of 63 solutes in RP-HPLC systems with the same mobile phase and 23 different commercially available packings. They used correspondence factor analysis to evaluate variations of selectivity with column type. The factors affecting solute selectivity were grouped into types: hydrophobic factor and chemical and/or steric factors.[58]

Olsen et al. determined five chromatographic properties (hydrophobic and free silanol interactions, trace metal activity, silanol interaction and shape selectivity) of 17 octadecylsilyl phases to examine column similarities and differences for column selection in method development. They used PCA and cluster analysis to analyze their data.[59] Hamoir et al. have used a PCA variant, namely spectral mapping analysis, for the characterization of stationary phases.[55]

Since 1998, column selectivity in RP-LC has been extensively studied by the group of Snyder.[20,60,61] Their hydrophobic-subtraction model describes that a solute retention factor k is related to the value of k for ethylbenzene (k_{ref}) and to parameters that depend on the solute (η', σ', β', α', κ') and column parameters: H (hydrophobicity), S^* (steric selectivity), A (hydrogen-bond acidity), B (hydrogen-bond basicity) and C (cation-exchange/ion interaction behavior). Snyder et al. have investigated Type-B alkyl-silica columns,[62] higher metal content (type-A) alkyl-silica columns,[63] columns with embedded or end-capping polar groups,[64] cyanopropyl columns[65] and Phenylalkyl and fluoro-substituted columns.[66] Values of H, S^*, etc. can be determined by carrying out retention measurements for 18 test solutes under standardized conditions. The reproducibility of the procedure has been evaluated by

comparison testing in four different laboratories and found acceptable. An alternative 10-solute test procedure, which is more reproducible and convenient (but somewhat less accurate), requires only 2–3 h per column.[67] This research group proposed an approach for choosing an equivalent replacement column for a RP-LC assay procedure.[9]

Cruz et al. characterized 30 different commercially available columns.[8] The dataset contained five chromatographic properties (amount of alkyl chains, hydrophobicity, steric selectivity, hydrogen-bonding capacity, ion-exchange capacity at pH > 7 and pH < 3) and also an efficiency parameter for each column was added. The column properties were graphically presented using adapted Tanaka radar plots.[35] PCA and cluster analysis were used to group columns having similar chromatographic properties. Euerby et al. increased the Cruz's dataset to 85 columns.[21] The classification was performed using PCA. They found by an initial PCA that three of the seven parameters correlated highly. Therefore only five parameters were included into the column characterization procedure. In 2003, Euerby et al. extended their database to 135 different stationary phases and employed PCA to classify them.[18] They explain the way to interpret PCA score and loading plots practically for comparing columns and also describe a method to identify similar columns without PCA using a spreadsheet program.

In 2005, they published results on a range of commercially available polar embedded columns and compared them to their C-alkyl analogues and to a range of "Aqua" and amino end-capped phases.[68] The column characterization protocols by Layne, Waters and Tanaka (modified by Euerby), when combined with the chemometrical tool of PCA, have been shown to be a powerful and easy way of discriminating between commercially available polyethylene glycol (PEG) phases, standard C-alkyl and amino end-capped phases.

Hoogmartens et al. chose eight rather simple, widely used methods for testing RP columns after careful consideration of the literature. After adaptation of these methods, a new test procedure was proposed, which allows one to examine all the important properties of the RP stationary phases such as efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and presence of metal impurities.[69] This test procedure was carried out on 69 different RP-LC columns and 36 chromatographic test parameters were evaluated overall. Several stationary phases were investigated in different laboratories in order to obtain reproducibility data. Each property of the RP stationary phases was characterized by different parameters. The different chromatographic parameters, their repeatability, reproducibility and correlation were critically studied.[19]

Twenty four parameters were selected out of 36 that could be measured in a repeatable and reproducible way by Visky et al.[54] PCA was performed on these 24 parameters to classify the columns. Different types of columns (stationary phases made of new/old type of silica gel,

normal/large pore size, end-capped/non–end-capped, base deactivated/not base deactivated and polar embedded) were distinguished with these tests. The aim of Hoogmartens's group was to reduce the number of parameters in order to develop a general but simple test procedure to characterise RP columns. Ivanyi et al. evaluated from Euerby's earlier dataset that PCA offers a possibility to reduce the number of parameters from five to four or even three due to their correlation while maintaining the classification.[28] Visky et al. applied the same chemometric tool to estimate the minimal number of parameters necessary for rational classification. It was shown that after carefully reducing the number of parameters from 24 to 4, similar classification was obtained. The remaining four chromatographic parameters can be determined with three reproducible methods.[54]

The next step was to verify that columns that have closely related characteristics (as determined by these chromatographic tests and classified into the same group) are suitable for the same chromatographic application. First, the separation of acetylsalicylic acid (aspirin, ASA) and related compounds was performed according to the Ph. Eur. monograph on the stationary phases, which were previously characterized chromatographically and classified. Results showed that the proposed classification of the columns is helpful. Columns with similar column selectivity for the separation of ASA and its related substances are situated in the same group. The system suitability test (SST), which is described in the Ph. Eur. in order to distinguish between suitable or unsuitable columns for this separation, was also evaluated. It was concluded that not all the columns, which meet the requirements of the SST, gave baseline separation.[70]

Hoogmartens et al. proposed a column ranking procedure, which is similar to Euerby's method, based on the four parameters mentioned earlier. One has to select a reference column (default reference is the Alltima C18) and the system ranks all the other columns in the order of similarity.[71] It is very easy to find a replacement column if the ideal column is not available. Orthogonal column selection is also possible with this database; one has to select one of the most dissimilar columns from the list. The system contains data for more than 80 columns and is freely accessible through the Internet.[72] Hoogmartens's group performed another six separations of drugs and their impurities on the same columns, which were characterized before and concluded that the ranking system facilitates the column selection practice.[23]

IV. CONCLUSIONS

In this chapter, the difficulty in RP-LC stationary phase selection has been highlighted. Manufacturers provide only limited information about the stationary phases and use different tests and evaluation parameters for their columns. Official compendia also do not mention the brand of

the stationary phase(s) that can be used in order to obtain sufficient selectivity during a given RP-LC analysis. Since more than 600 different RP-LC columns are available on the market, it is extremely difficult to select the optimal column for a certain application, and to find a replacement or orthogonal chromatographic support.

The most important characteristics of RP-HPLC columns, which play an important role in real separations, are reviewed. These characteristics (efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the amount of metal impurities) can be determined with different chromatographic test methods. These test procedures, the evaluation of the parameters and their interpretation are summarized. Commercially available test mixtures and their practical application are also discussed. Such chromatographic test procedure can also be used to check the performance of a column at any time of its life cycle.

As was discussed here, various research groups performed chromatographic test approaches on several RP columns. The parameters, which are evaluated from the results of the chromatographic test methods and resulted in huge databases, can be used as an excellent source to classify RP-HPLC columns according to their performance. Chemometric tools such as PCA were applied to facilitate the interpretation of these large databases and classification of stationary phases. The existing databases, which offer great practical assistance in selection of appropriate RP-HPLC column(s), were also reviewed. One of these databases is freely accessible through the Internet.

It can be concluded that chromatographic characterization approaches combined with different chemometric tools are useful in identification of columns with similar properties. This eliminates the need to be tied to one manufacturer and gives an opportunity to choose replacement columns easily. Selection of columns, which differ in chromatographic characteristics and can be used for orthogonal method development, is also less problematic. It is also possible to obtain a greater understanding of the molecular interactions between the analyte and the stationary phase.

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CHIRAL SEPARATIONS

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ABSTRACT

This chapter discusses chiral separation of active pharmaceutical ingredients (APIs) and their related compounds by various methods. Particular emphases have been put on chiral separation by HPLC on chiral stationary phases (CSPs). Different types of CSPs, including polysaccharide derivatives, macrocyclic glycopeptides (antibiotics), cyclodextrins, Pirkle-type, proteins, ligand exchange, crown ethers, synthetic polymers/molecularly imprinted

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polymers (MIPs), are discussed. Separation mechanisms and method development for chiral molecules using these phases are also discussed.

I. INTRODUCTION

A. General

The detection and quantitation of optically active impurities in chiral active pharmaceutical ingredients (APIs) and drug products remain challenging in pharmaceutical analyses. The regulatory authorities have encouraged the pharmaceutical industry to treat multiple stereoisomers of a chiral API as separate entities because of the potential differences in pharmacological activities. The industry has responded with an increased number of filings for single enantiomeric drugs. In support of these filings, chiral analytical methods are required for quality control of the drug substance and product. Furthermore, it has been emphasized that sensitive and reliable chiral analytical methods should be implemented in early-phase drug development to understand the fate of the API in vivo, as well as to provide justifications for setting specification limits.[1]

Various methods including gas chromatography/liquid chromatography (GC/LC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), and capillary electrochromatography (CEC) have been used in chiral separation of APIs. However, in pharmaceutical analysis, particularly in later-phase quality control of APIs and products, the majority of the chiral methods are developed by HPLC with chiral stationary phases (CSPs). Topics related to chiral HPLC methods are the focus of this chapter.

B. Overview of Chiral Separation Techniques

Before we discuss the use of HPLC with CSPs, a brief overview for the other methods that may find applications in chiral separation of APIs and related compounds is warranted.

1. Chiral Separation by Gas Chromatography

The first successful separation of enantiomers by chiral GC with a CSP was reported in the 1960s.[2] Since then, many different types of GC CSPs have been reported in the literature and some of them have been commercialized. The most important GC CSPs are derivatized cyclodextrins.[3] Chiral GC has the advantages of simplicity, speed, high efficiency, and accuracy. However, chiral GC is more suitable for analytes that are relatively volatile. For example, many racemic alcohols, amino alcohols, amines, lactones, and halohydrocarbons have been separated on GC CSPs. These classes of compounds may be important building blocks in the synthesis of chiral APIs and are difficult to separate by LC. Chiral GC may find applications in the

analysis of chiral starting materials and intermediates in the synthesis of chiral APIs. It has rarely been used for the analysis of chiral APIs simply because they are not volatile or would not survive the high temperature.

2. Chiral Separation by Supercritical Fluid Chromatography

Chiral separation by SFC was first reported in 1985.[4] In the SFC mode, some of the chiral HPLC columns (even some GC columns) can be used directly without modification.[5] Compared with HPLC, one of the unique characteristics of SFC is its supercritical mobile phase, which can be pumped through the column at higher flow rate. In some cases, because of its unique chemical properties, the mobile phase may offer unique chiral selectivity. One drawback of SFC is that the observed peak shapes may be not as smooth and symmetrical as those obtained in normal-phase HPLC. This effect may result from the changes in density and viscosity of the super/subcritical fluid due to the SFC operating conditions (i.e. temperature and pressure, etc.), which in turn may cause changes in solubility of the analyte in mobile phase over the length of column. Another drawback is the poor detection sensitivity in the SFC mode. Because of these drawbacks, chiral SFC has not found wide applications in analytical scale separation of APIs. However, chiral SFC is quite popular in preparative scale separation because the collected fractions can be easily recovered due to the volatility and inertness of the mobile phase.

3. Chiral Separation by Capillary Electrophoresis

Chiral CE methods are complementary to chiral HPLC methods in pharmaceutical analyses. Inherently, CE methods are more suitable for polar and charged molecules whereas HPLC methods work the best with relatively nonpolar and neutral molecules. Chiral separation by CE is achieved by addition of chiral selectors to the running buffer. Many of the CSPs for HPLC can be used as chiral selectors for CE, including polysaccharide derivatives,[6] macrocyclic glycopeptides (antibiotics),[7–9] cyclodextrins,[10–12] proteins,[13] and polymers/molecularly imprinted polymers (MIPs). CE is particularly useful in early-phase drug development because of its high separation efficiency, high selectivity, and low consumption of solvents/chiral selectors. With CE, it is also practical to use a combination of multiple chiral selectors in a single run. The method development process is usually faster than LC because the analyst can screen multiple chiral selectors quickly. In contrast, chiral HPLC methods may be more suitable for later-phase methods when method robustness and transferability become more important. Because the focus of this chapter is on chiral separation by HPLC, the reader is referred to the literature for details regarding chiral CE applications in chiral drug analysis.[14–16]

4. Chiral Separation by Capillary Electrochromatography

The CEC technique, which was developed in early 1990s,[17] is a hybrid of chiral HPLC and CE. CEC is expected to combine the high

peak efficiency characteristic of electrically driven separations with the high separation selectivity of multivariate CSPs available in HPLC. Although chiral CEC has been successfully applied to enantioseparation of chiral analytes of different structures, it has not found many applications in the pharmaceutical industry. The main reason is that packing a capillary with silica-based phases is not easy and the preparation of frits by sintering a zone of packing still remains a sophisticated procedure. A lot of recent research has been performed in the field of monolithic fritless CEC phases.[18] Successful commercialization of such techniques could boost CEC application in pharmaceutical industry.

5. Chiral Separations by HPLC

Chiral separation of enantiomers, including APIs, can be achieved through three general approaches by HPLC. The first approach utilizes enantiomerically pure chiral derivatizing agents to convert the enantiomers into diastereomers, which then can be separated using achiral HPLC. Chiral derivatization was widely used in the 1970s and early 1980s but is now less favored because it is time consuming and may cause racemization and/or different reaction yields of the diastereomers.[19]

The second approach uses chiral mobile-phase additives (CMPAs). CMPA, sometimes in the presence of a third component (such as a metal ion), will form transient diastereomeric complexes. Then, achiral HPLC is used to separate the complexes. This approach requires a continued supply of often-expensive CMPA. In addition, CMPA may limit the use of detection methods. Additional disadvantages for this approach may also include poor peak shapes and low column efficiency.[20,21]

The third approach, which is the predominantly used approach in pharmaceutical analysis, involves chemically bonded or coated CSPs. The CSP interacts with analyte enantiomers dynamically to form short-lived, transient diastereomeric association or complexation. The intermolecular interactions between CSP and the analyte include hydrogen bonding, π–π interaction, dipole stacking, inclusion complexing, steric interaction, ionic interaction, etc. Each of these interactions will exhibit different strength under different separations modes. In order to achieve chiral separation, a three-point interaction is necessary and at least one of the three points has to be stereospecific.[22]

Chiral separation methods on CSPs by HPLC can be developed in three different modes: reversed-phase, normal-phase, and polar organic mode. Reversed-phase separation uses aqueous mobile phase with organic modifier. Normal-phase separation utilizes nonpolar organic solvent such as hexane or heptane with polar organic modifier. The polar organic mode was first used on cyclodextrin CSP by Armstrong et al.[23] In this mode, polar organic solvents such as acetonitrile and methanol are used as the main component of mobile phase. Small amounts of base and/or acid such as triethylamine (TEA) and acetic

acid (AA) are added as modifiers. Since then, the term "polar organic mode" has been widely accepted and extended to all CSPs that use similar mobile phases.

II. SEPARATION OF ENANTIOMERS ON HPLC CHIRAL STATIONARY PHASES

Since the first patent was filed in 1968 on an HPLC CSP based on ligand-exchange chromatography (LEC),[24] more than 1300 CSPs of various types have been reported and approximately 170 of them are commercially available.[25] Among all the commercial CSPs, there have always been a few dominant ones that accomplished the majority of separations. Ligand-exchange CSPs were the leading products before the early 1980s.[26] In 1985, three of the most popular columns were based on 3,5-dinitrobenzoylphenyl-glycine (Pirkle-type), β-cyclodextrin, and α_1-acid glycoprotein (AGP).[27–29] By 1990, 3,5-dimethylphenylcarbamated cellulose columns proved to be the most widely applicable and crown ether columns showed broad selectivity and ease of use for amino acid separations.[30,31] By 1995, macrocyclic glycopeptide columns were having a significant impact because of their wide utility and high efficiency.[7,32,33] Around this time, a new Pirkle-type column (Whelk-O 1) and a widely useful derivatized amylose were introduced.[34,35]

Various types of CSPs have played important roles in the development of novel chromatographic systems and in evaluating the chiral recognition mechanism. However, driven by industrial chemists for cost-effective method development, the majority of chiral pharmaceuticals have been separated on either polysaccharide or macrocyclic glycopeptide CSPs. These two phases are categorized as universal CSPs. The polysaccharide-based CSPs that are commercially available include Chiralcel OD, OD-H, Chiralpak AD, AD-H, Chiralcel OJ, OJ-H, and Chiralpak AS, AS-H, which are manufactured by Daicel Chemical Industries in Japan. The macrocyclic glycopeptide (antibiotics) CSPs include Chirobiotic V, VAG (vancomycin), Chirobiotic T, TAG (teicoplanin) and Chirobiotic R (ristocetin A) that are manufactured by Astec in New Jersey. In the following sections, the two universal types of CSPs are discussed in terms of chiral recognition mechanism and method development strategy. Other major types of CSPs are also discussed but in less detail.

A. Polysaccharide-Based CSPs

1. Introduction

Polysaccharides such as cellulose and amylose are among the most abundant optically active biopolymers. After derivatization, as first

achieved by Hesse and Hagel in 1973,[36] they can resolve many aromatic and aliphatic enantiomers. In 1984, Okamoto et al. prepared a CSP by coating tris(phenylcarbamate) derivatives of polysaccharide on macroporous γ-aminopropylsilica.[37,38] Among the prepared derivatives, cellulose and amylose tris(3,5-dimethylphenylcarbamate) showed particularly interesting and excellent resolving ability for a variety of racemic compounds and they were commercialized under trade names Chiralcel OD and Chiralpak AD, respectively.

Since the derivatized polysaccharides can be dissolved or swollen in solvents such as chloroform or tetrahydrofuran, the use of certain solvents in mobile phase is prohibited on the coated CSPs. In an effort to overcome this shortcoming, tris(3,5-dimethylphenylcarbamate) of cellulose and amylose were regioselectively bonded to silica gel.[39] A variety of eluents can be used in chiral HPLC using the bonded-type CSPs. However, the bonded CSPs show slightly lower enantioselectivity than the coated ones. Some derivatives, such as the halogeno-phenylcarbamate derivatives, are soluble in typical normal-phase organic solvents. Photochemical techniques have been applied to immobilize CSPs to silica-coated materials.[40] Some of the immobilized columns are commercialized. The immobilized phases were found to exhibit unique resolution compared with conventional coated polysaccharide-based CSPs.[41] However, coated phases still dominate the polysaccharide CSP market. Caution needs to be practiced in the use, maintenance, and storage of the coated columns to ensure robust methods and long column life. Users should refer to the manufacturer's column instructions as to the column pressure, flow rate, washing procedure, and compatible solvents.

2. Chiral Recognition Mechanism

The chiral recognition mechanism of polysaccharide-based CSPs has been investigated extensively, usually based on chromatographic methods.[42] Spectroscopic approaches including NMR and MS combined with computer modeling have also been applied.[43–45] In a structural model study, Vogt proposed that tris(phenylcarbamate) cellulose possesses a left-handed threefold (3/2) helix and tris(phenylcarbamate) amylose has a 4/1 helical conformation.[46,47] As shown in Figure 1, the glucose residues on these derivatives are regularly arranged along the helical axis. A chiral helical groove with polar carbamate residues exists along the main chain. The polar carbamate groups are located inside, and hydrophobic aromatic groups outside, the polymer chain. Therefore, enantiomers coming from outside the groove can effectively interact with the polar carbamate groups via hydrogen bonding with the NH and C=O groups and dipole–dipole interaction on the C=O. The nature of the substituents on the phenyl groups affects the polarity of the carbamate residues, which leads to different chiral selectivity. Besides these polar interactions, π–π interactions between the phenyl group of a CSP and an aromatic group of the solute

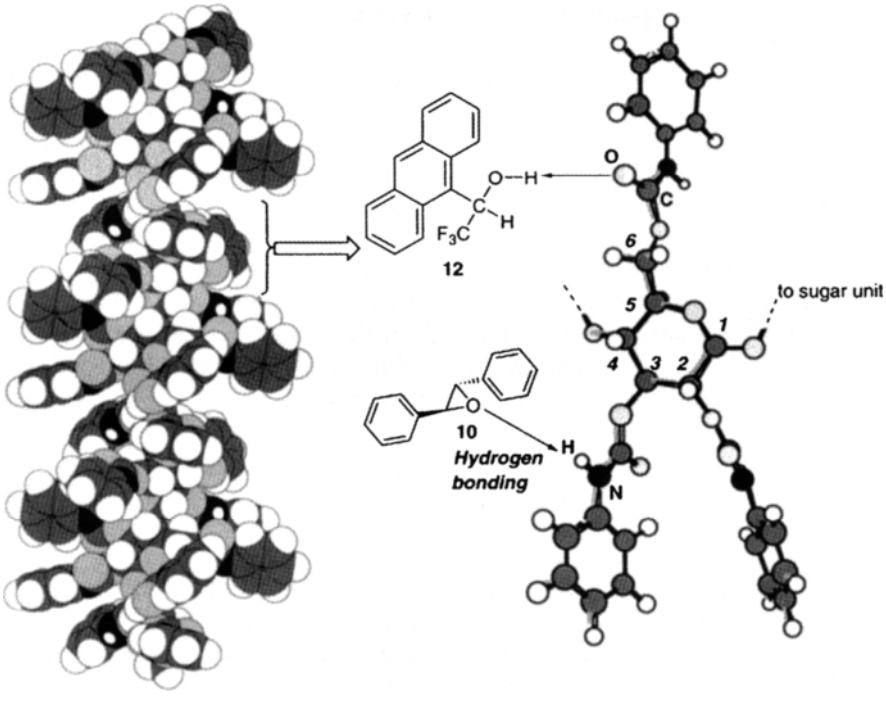

FIGURE 1 **Optimized structure (left) and possible interaction sites (right) of cellulose tris(phenylcarbamates) (chiral selector in Chiralcel OD, reproduced with permission from reference 46).**

may play some role in chiral recognition, particularly in the reversed-phase mode of HPLC separation. The different helical nature of amylose and cellulose derivatives allows different inclusion into the phase and different chiral recognition possibilities. Both CSPs were found to be very sensitive to the molecular size adjacent to the asymmetric center. Increasing aromatic bulk may lead to decreasing chiral recognition ability.

3. Method Development

Initially, the polysaccharide type CSPs (Chiralpak AD, Chiralcel OD, etc.) were mostly used with normal-phase eluents, since interactions such as hydrogen bonding, π–π interaction, and dipole–dipole stacking interaction are considered to be more effective under normal-phase conditions. Later, they were proved useful in the polar organic mode and reversed-phase mode.

(a) Normal-Phase/Polar Organic Mode:

(i) Organic Modifier. The mobile-phase composition under normal-phase conditions is usually hexane/alcohol. A typical mobile phase in

the polar organic mode contains alcohol/acetonitrile. Among all alcohols, isopropanol and ethanol are commonly used in normal phase while methanol and ethanol are normally used in the polar organic mode. In both modes, alcohol is the strong solvent. Increased concentration of alcohol leads to decreased retention. As far as enantioselectivity is concerned, it is difficult to predict which solvent is the most favorable modifier in order to achieve optimal separation. Retention order may be altered by changing mobile-phase modifiers. This is illustrated by the effect of alcoholic modifier on retention time of an API that has 16 stereoisomers (or 8 pairs of enantiomers). When IPA was changed to ethanol, the elution pattern for the isomers was significantly altered (Figure 2).[48] In some cases where multiple stereoisomers need to be resolved from each other, two or more alcoholic modifiers are used to achieve desired separation.

(ii) Acidic/Basic Additives. Acidic/basic additives such as trifluoroacetic acid (TFA), AA, diethylamine (DEA), or TEA are usually added to the mobile phase when the analyte is acidic/basic. The additives normally sharpen the peaks and improve resolution. Other acids, such as methane sulfonic acid, have also been used as acid additives for the enantioseparation of various compounds including amino acids.[49] One example was the enantiomeric separation of ofloxacin. Typical acidic additives such as AA, TFA can only result in partial separations. Baseline

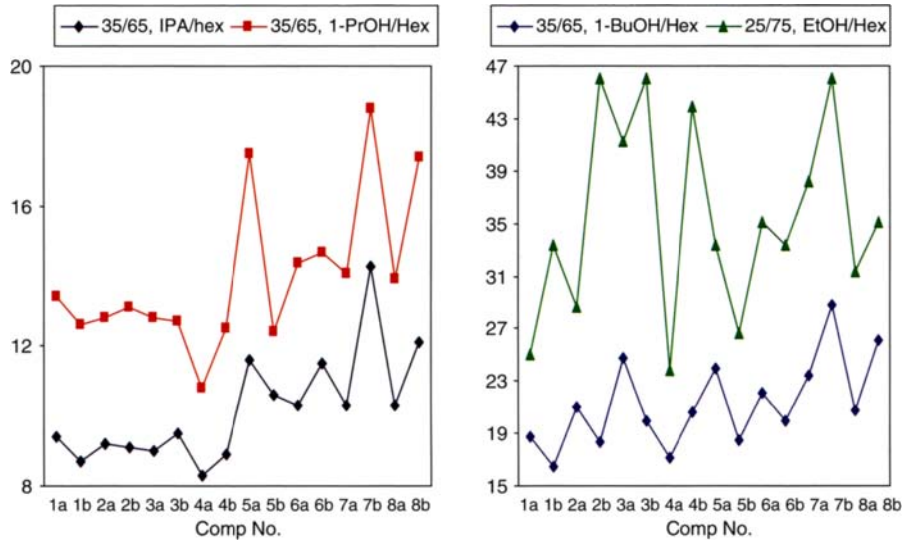

FIGURE 2 **Effect of organic modifier on the retention time (min) of 16 stereoisomers of an API (1a–8a are the enantiomers of 1b–8b). (Column) Chiralpak AD-H, 250 × 4.6 mm, 5 μm, flow rate 0.6 mL/min, column temperature 35°C.**

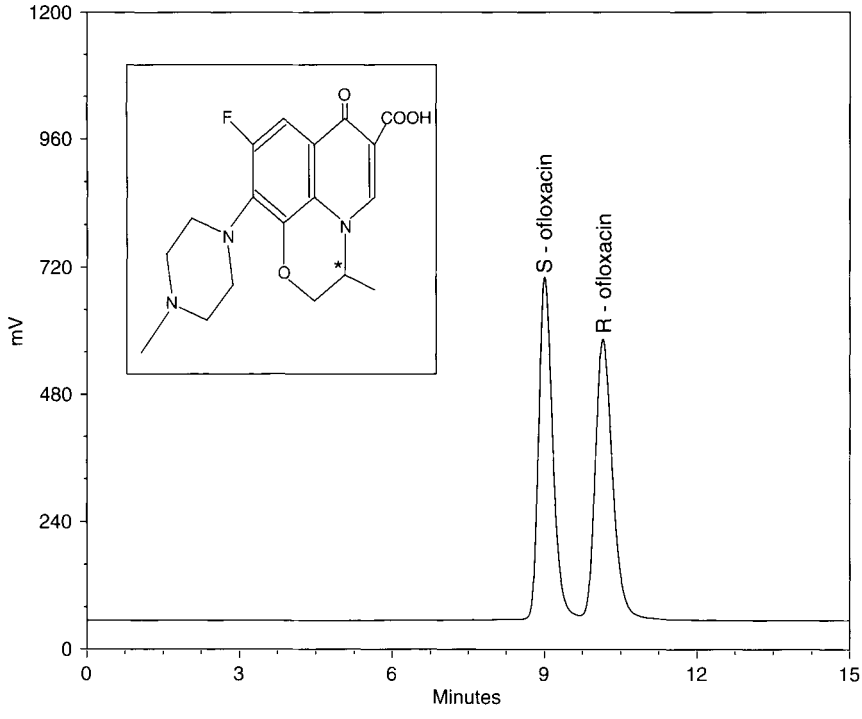

FIGURE 3 **Enantiomeric separation of ofloxacin. (Column) Chiralcel OD-H, mobile phase: 95/5/0.1/0.025, EtOH/MeOH/$MeSO_3H$/DEA, flow rate 0.5 mL/min, temperature 30°C, UV detection at 273 nm.**

separation was solely achieved by using methane sulfonic acid as the additive (Figure 3).[50] Some cyclopropylamine and cyclobutylamine can significantly enhance enantioselectivity for a variety of phenylamine analogs.[51]

(iii) Water. The presence of water in the mobile phase affects hydrogen bonding, which is one of the important interactions between the analyte and the CSP. In certain instances and for difficult separations, it may be crucial to control the water content in the mobile phase. Increasing the water content could change the retention of enantiomers or inverse the retention order.[52]

(iv) Temperature. For some applications, especially difficult chiral separations, the effect of temperature on separation should be studied. Normally, enantioselectivity favors low temperature while the column efficiency tends to be better at higher temperature. An optimized temperature is often a compromise.[53,54] When temperature is not critical to separation, 35°C is recommended as a column friendly temperature and is desirable for robust and easy-to-transfer methods.

(b) Reversed-Phase Mode:

The first chiral separation using an aqueous buffer on polysaccharide type CSPs was reported by Ikeda et al. in 1989.[55] There are a number of advantages to use the reversed-phase conditions, for example, good solubility of polar compounds, easier sample preparation from serum, plasma or many drug products, and use of less costly solvents.

(i) Organic Modifier. It is common to use either acetonitrile or methanol as an organic modifier under reversed-phase conditions. The retention time of an analyte can be decreased by increasing the amount of the organic modifier. In addition, the same acetonitrile concentration usually gives a shorter retention time compared with methanol.[56] However, there is no rule as to the choice of a polar organic modifier for better peak shape.

(ii) Temperature. The effect of temperature on reversed-phase separation is similar to normal-phase/polar organic modes.[57]

(iii) Acidic/Basic Additives. When using the polysaccharide CSPs, it is important to keep the analyte neutral in order to maximize the interaction between the CSP and the analyte. Acidic or basic modifiers have minimal effects on a neutral analyte. For an acidic analyte, however, it is essential to use an acidic mobile phase. For a basic analyte, two parameters need to be considered: the choice of a buffer system and a suitable pH. A borate buffer around pH 9 is a good starting point, whereas a pH of less than 7 is preferred to preserve column lifetime. The retention and resolution of basic analytes are influenced considerably by the type of anions added to the mobile phase. In addition to borate buffer, KPF_6, $NaPF_4$, $NaPF_3SO_3$, $NaCF_3SO_3$, $NaClO_4$, $NaNO_6$, NaH_2PO_4 are among the commonly used additives. Small amounts of organic amines (e.g. 0.1–0.2% of DEA) may be added to improve the peak shape. For onium ion analytes, such as quaternary amines, it is essential to use an ion-pair reagent. While higher concentration generally helps with separation, it is suggested that buffer concentrations be at less than 50% of the saturation level of the salt to minimize potential precipitation.[58,59]

B. Macrocyclic Glycopeptide (Antibiotics) CSPs

1. Introduction

The macrocyclic glycopeptide antibiotics consist of an aglycon portion of fused macrocyclic rings that form a characteristic "basket" shape and carbohydrate moieties attached to the aglycon basket. The aglycon basket consists of three or four fused macrocyclic rings composed of linked amino acids and substituted phenols. The carbohydrate moieties consist of carbohydrate or saccharide groups. The glycopeptide antibiotics differ in the number and type of pendant carbohydrate groups. The sugar groups

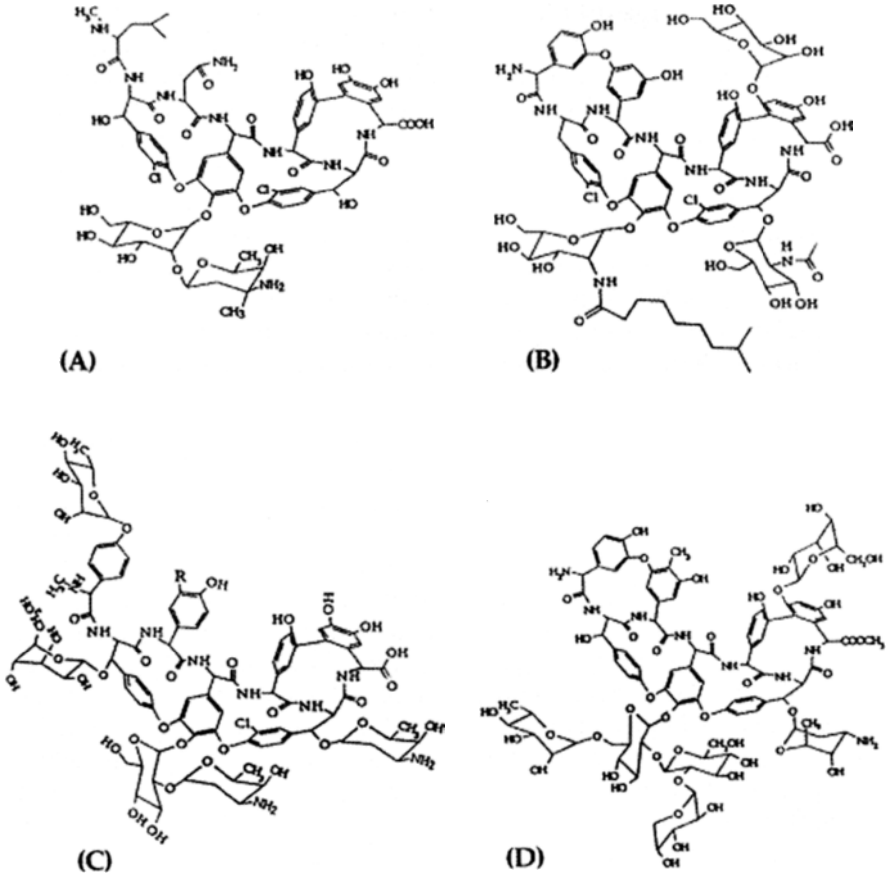

FIGURE 4 Chemical structures of the macrocyclic glycopeptide antibiotics: (A) vancomycin (Chirobiotic V), (B) teicoplanin (Chirobiotic T), (C) avoparcin, and (D) ristocetin A (Chirobiotic R). (Reproduced with permission from reference 60.)

attached to the aglycon basket are free to rotate and can assume a variety of orientations (Figure 4).[60] The sugar units can be removed through chemical reactions.[61] Both the original glycopeptides and the sugar-removed ones are covalently bonded to silica gel. Armstrong et al. have commercialized these under the trade names: Chirobiotic V, VAG (vancomycin), T, TAG (teicoplanin), and R.[62]

The unique structure of the macrocyclic glycopeptides and their abundance of functionality (e.g. aromatic, hydroxyl, amine, and carboxylic acid moieties, amide linkages, hydrophobic pockets, etc.) give them broad selectivity for a wide variety of anionic, neutral, and cationic compounds. All of the defined molecular interactions that are necessary for chiral recognition are found within these relatively compact structures.

Macrocyclic glycopeptide stationary phases are multimodal stationary phases that display the following qualities: (i) the stationary phases are stable in most solvents used; (ii) any changes in the stationary phase (conformation of selector) are reversible in response to changes between chromatographic modes; (iii) there are significant selectivity and mechanistic differences in each mode; (iv) there are logical or systematic approaches for selecting a particular mode of separation and optimization. Macrocyclic glycopeptide CSPs can be effective in three modes of chromatographic operation and each mode has a distinct mechanism of chiral recognition.

2. Chiral Recognition Mechanism

(a) Reversed-Phase Mode:

In the reversed-phase mode, as with achiral HPLC stationary phases, the glycopeptide CSP is relatively nonpolar compared with the polar hydro-organic mobile phase. Hydrophobic interaction between the nonpolar part of an analyte and the interior of the glycopeptide basket is a dominant factor in retention and may also be one of the important factors contributing to chiral recognition.[7,32,33,63,64] Additional interactions that can lead to chiral recognition include electrostatic interactions, hydrogen bonding, dipole–dipole interaction, and steric repulsive interactions. Which interactions are most important for a specific compound depends on the nature of that analyte (i.e. its size, geometry, number and type of functional groups, flexibility, etc.) as well as the nature of the mobile phase.

Electrostatic interactions can affect both retention and enantioselectivity. It was observed that when an amine group of a chiral analyte is protonated at low pH, retention is significantly increased. However, the selectivity factor (α) decreased somewhat. This reveals that the electrostatic interaction between the carboxylate group on the CSPs and the amine group on the analyte is effective only for increasing retention, but not for enhancing chiral recognition of enantiomers. In contrast, it was verified that interaction between the amine moiety of macrocyclic glycopeptide CSP and the carboxylate group of the analyte play a crucial role in the chiral recognition mechanism.[64,65] This was further supported by the fact that molecules with acidic groups (i.e. carboxylate, sulfinate, phosphate, etc.) are most easily resolved on macrocyclic glycopeptide CSPs.[66,67]

(b) Normal-Phase Mode:

In the normal phase mode, the polar functional groups and aromatic rings of the CSP provide the interactions needed for both retention and chiral recognition. Therefore, hydrogen bonding, π–π interactions, dipole stacking, steric repulsion, and in some cases, electrostatic interactions are the dominant interactions that occur between the CSP and the

analytes. Note that the absence of water diminishes the usefulness of hydrophobic interactions in chiral recognition in this mode. However, in the presence of nonpolar solvents, the enhanced π–π and dipolar interactions often make up for the lost contribution from hydrophobic interactions that were important in the reversed-phase mode.[68]

(c) Polar Organic Mode:

In the polar organic mode, the main mobile-phase component is an alcohol (e.g. methanol, ethanol, or isopropanol). The dominant interactions between the analyte and CSP usually involve hydrogen bonding, electrostatic, dipolar and steric interactions (or some combination thereof). Chiral analytes suitable for this mode should have at least two polar functional groups. These groups include alcohols, halogens, nitrogen in any form (primary, secondary, and tertiary amines), carbonyl, carboxyl, oxidized forms of sulfur and phosphorus, for example. At least one of the analyte's polar functional groups must be on or near the stereogenic center. It is also beneficial if the analyte has some steric bulk or aromatic rings close to the stereogenic center.[69]

3. Method Development

Given the variety of the functionality that exists within the macrocyclic glycopeptides, Chirobiotic columns can work well in reversed-phase, normal-phase, and polar organic modes. Most of the time, higher efficiencies are observed in the polar organic and normal-phase mode. The ability to operate in different modes can be advantageous, since different compounds separate best in different modes. If the polar organic modes is applicable, it is usually the best choice to start with. Solubility of the analytes in different solvents also affects the choice of mobile phase.

(a) Reversed Phase:

(i) Organic Modifier. Typically, a U-shaped curve is observed when plotting retention and resolution vs. composition of organic modifier/aqueous solution. Longer retention and better resolution are usually observed in both the high and low concentration regions of organic modifier.[68] This retention behavior clearly indicates the importance of hydrophobic interactions between the analyte and the macrocycle at higher concentrations of aqueous buffer. When using higher concentration of organic modifier, hydrophobic interactions no longer contribute to retention. However, other interactions become increasingly dominant (e.g. hydrogen bonding, dipolar interactions, etc.).

The type of organic modifier can affect both selectivity and efficiency. Different CSPs prefer different types of organic modifiers. For example, tetrahydrofuran and acetonitrile work best on Chirobiotic V, while alcohol-type modifiers works best on Chirobiotic T and R columns. A typical starting composition is 10:90, MeCN/buffer, pH 3.5–7.0 or 20/80, methanol/buffer.[67]

The retention behavior of amino acids is different from that of most other analytes. Amino acids often have smaller retention factors with water-rich mobile phases. This phenomenon may be due to the higher solubility of amino acids in water than almost all other solvents. In addition, it has been shown that the electrostatic interaction between the amino acid and CSP is so strong that organic modifier concentration does not affect the retention and selectivity factor as much as it does other analytes. The resolution factor does change with the mobile-phase composition.[32]

(ii) Buffer. Buffer is widely used in the mobile phase for macrocyclic glycopeptide columns to control ionization of both analyte and the CSP. In addition, buffer has ionic strength effects and affects the selectivity for certain compounds via secondary interactions between the buffer and CSP and/or buffer and analyte. Use of buffer can increase efficiency of the separation significantly. To obtain efficient and reproducible separations in the reversed-phase mode, buffer is essential even for separations of neutral compounds.[69] The recommended buffers in the reversed-phase mode in order of their usefulness are triethylammonium acetate (TEAA), ammonium acetate, ammonium nitrate, and sodium citrate. Percentage of the buffer salts can be varied from 0.01% to 1% depending on the retention factor of specific analytes. Normally, the higher the buffer concentration, the shorter is the observed retention. Buffer solutions with 0.1% TEAA are most frequently used. They are prepared by titrating a 0.1% solution of TEA with AA to the appropriate pH.

Macrocyclic glycopeptides have ionizable groups as proteins do, therefore, their charge and perhaps their conformation can vary with pH of the mobile phase. pH will have different effects with different chirobiotic columns because of variations in their functional groups and their pI value. Within the operating pH range (3.5–7.5), the strength of the short-lived complexes formed by the CSP and the analyte can depend on the charge of the analyte. The charge of the analyte will in turn be affected by pH of the mobile phase. Vancomycin and ristocetin A are both in cationic form, while teicoplanin and most amino acid analytes exist in the zwitterionic form (i.e. with an anionic $-COO^-$ group and a cationic $-NH_3^+$ group). Changes in pH of the mobile phase can change ionization of both the analyte and the CSP. Therefore, pH can affect the interaction mechanism even if the analyte is a neutral molecule. Generally, the starting pH should be close to the pI value of the glycopeptides. Alternatively, test runs can be made at pH 4 and 7. After finding which pH produces the optimum separation, the pH can then be adjusted to 0.5 pH unit above or below the pK_a of the analyte.[67]

(iii) Temperature. Temperature can affect the retention and resolution probably by affecting the binding constant of a solute to the CSP. Retention factor, separation factor, and column efficiency are all

affected by temperature. Temperature and solvent composition are independent in terms of their effects toward the above factors. Usually an increased resolution can be achieved by lowering the separation temperature.[70,71]

There are two different temperature-related effects on enantiomeric resolution. One is the thermodynamic effect that is responsible for the change in the selectivity factor (α) when temperature is varied. This is due to the partition coefficients and therefore, the Gibbs free energy change of transfer of the analyte between the mobile phase and the stationary phase. The other is the kinetic effect that produces changes in column efficiency with varied temperatures. This results from the decease in viscosity and the increase in the analytes' diffusion coefficients.

(iv) Flow Rates. Usually, flow rate does not affect the enantioselectivity factor (α), but does affect the separation efficiency. Flow rates <0.5 mL/min are not very common, since it will not produce further significant increase in resolution. A flow rate of 0.5–1.5 mL/min is recommended.

(b) Normal Phase:

In normal phase, there is no solvent-induced denaturation or any irreversible change in these CSPs. The advantage of using Chirobiotic columns under normal phase conditions is that they have higher efficiency and the selectivity is often complementary to reversed phase. In normal-phase HPLC, hydrogen bonding, π–π interactions, dipole–dipole stacking, and steric interactions are more important.

(i) Organic Modifier. Retention is decreased as the percentage of organic modifier is increased. However, the exact effect of the mobile-phase composition varies somewhat from compound to compound. In addition, it has been observed that resolution could be dramatically changed with different modifiers. Usually this results from a change in column efficiency. For example, ethanol usually provides better efficiency than isopropanol. The most common organic modifiers are alcohols. Solvents as well as DMF and dioxane are also used.[62]

(ii) Other. Small amounts of acid and/or base can be added to optimize the separation. Temperature has a greater effect on the retention of solutes in normal phase than in reversed phase. No observable effects were shown on selectivity by increasing the flow rate up to three times. High flow rates (2–5 mL/min) can be used to shorten the run time.

(c) Polar Organic Mode:

In the polar organic mode, the main component of a mobile phase is alcohol (methanol, ethanol, or isopropanol) with a very small amount of acid/base added to affect retention and selectivity. This mobile phase is considered as an extreme case of normal phase (or reversed phase). Acetonitrile can be added to increase retention. There are usually two different factors that need to be adjusted to optimize a separation. The

first one is the absolute amount of acid and base added, which is essential for optimization of retention. The second is the relative ratio of acid to base, which controls the selectivity. The effect on temperature is similar to that in normal phase and flow rate has a smaller effect on selectivity than in the reversed phase.[68]

C. Cyclodextrin-Based CSPs

Cyclodextrins were first used in 1959 for chiral separations as selective precipitation/crystallization agents for enantiomers.[72] Later, they were used as mobile-phase additives in TLC to separate isomeric compounds.[73] The first high coverage and stable-bonded cyclodextrin CSP was developed by Armstrong and DeMond.[74] It was composed of β-cyclodextrin bonded to silica via a "6–10" unit spacer arm free of nitrogen. This CSP was commercialized in late 1983 by Astec (Cyclobond I).

The utility of cyclodextrin-based CSPs was further extended to α- and γ-cyclodextrin and a variety of derivatives, immobilized on a chromatographic support. Since 1988, several derivatized cyclodextrin CSPs have been developed in Armstrong's research group. These include: (1) acetylated cyclodextrin that offers unique opportunities in the normal-phase mode (Cyclobond AC);[75] (2) *S*- or racemic 3-hydroxypropylated cyclodextrin that generates extra chiral centers and/or hydroxyl groups located away from the mouth of cyclodextrin, which allow the bulky enantiomers to interact with the introduced functionality (Cyclobond SP, RSP);[76] (3) the (*R*)- or (*S*)-naphthylethylated cyclodextrin that utilizes the π-basic naphthyl ring to interact with any π-acidic analyte to create a π-electron transfer in the vicinity of a stereogenic center or offers the hydrogen donor or acceptor sites of the carbamate structure for stereoselective interaction (Cyclobond RN, SN);[77] (4) 3,5-dimethylphenyl carbamated cyclodextrin (Cyclobond DMP);[78] (5) dimethylated cyclodextrin CSP (Cyclobond DM);[79] and (6) *para*-toluoyl esterified cyclodextrin stationary phases.[80] Charged groups, such as sulfate, also have been introduced into cyclodextrins.[77] As a result, the CSPs offer not only inclusion complexation but also potential ionophoric moieties for electrostatic interactions. The possible optimization parameters (e.g. pH, ionic strength) for chiral separation development were increased. The introduction of electrostatic interactions through charged groups allows differentiation for analytes containing amines and other hydrogen-bonding groups.

Like macrocyclic glycopeptide CSPs, cyclodextrin-based stationary phases are considered to be multimodal stationary phases.[81–83] These CSPs can be effective in three modes of chromatographic operation and each mode has a distinct mechanism of chiral recognition.

1. Reversed Phase

In reversed-phase chromatography using aqueous mobile phases, chiral separations are achieved by the formation of enantioselective inclusion complexes. For an analyte molecule that is complexed within the cyclodextrin, both hydrogen bonding and steric interaction can occur between the secondary hydroxyls and substituents about the stereogenic center, providing the necessary three points of interaction that leads to chiral recognition. Alternatively, if the secondary hydroxyl groups are derivatized, additional interactions (steric repulsion, H-bonding, dipolar, π–π complexation, etc.) can occur.[84]

Some essential characteristics for a molecule to be separated in the reversed-phase mode include the presence of a hydrophobic group (such as an aromatic ring) for the formation of an inclusion complex. Furthermore, the aromatic group must be of a good size match to the cyclodextrin cavity. A second necessary characteristic is that the stereogenic center be located near one of the secondary hydroxyls at the mouth of the cyclodextrin cavity. Interaction with the cyclodextrin hydroxyl or derivative groups is usually the only way to obtain the additional simultaneous interactions necessary for chiral recognition.[84]

In the reversed-phase mode, the most commonly used organic modifiers are methanol and acetonitrile; less commonly used are tetrahydrofuran, isopropanol, and ethanol. Changes in enantioselectivity and efficiency can occur upon changing from one organic modifier to another. It is difficult to predict which modifier will produce the best results. However, it is similar to achiral chromatography in that acetonitrile tends to be a stronger eluent than methanol and that gradients can be performed on cyclodextrin CSPs.[82]

pH is important for reversed-phase separations. The polarity and charge of acidic and basic analytes are affected by pH that will in turn affect the solute's interaction with the CSP. It is more common that neutral analytes partition more strongly to the hydrophobic cyclodextrin cavity than their charged counterparts. Cyclodextrin CSPs are stable at pH 4.0–7.0.[82]

The nature and concentration of buffer is also important. TEAA buffer is preferred for most cyclodextrin CSPs because it is noncorrosive and enhances efficiency by masking available silanols and other strong adsorption sites. Other buffers that have been used on cyclodextrin CSPs include ammonium nitrate, ammonium acetate, citrate, and triethylammonium phosphate. Organic buffers are capable of forming an inclusion complex with the cyclodextrin cavity. Therefore, higher concentration often reduces retention. Additionally, most acid and base modifiers can compete for hydrogen-bonding sites on the cyclodextrin molecule. At high concentration (>1.5%), enantioselectivity can be altered, diminished, or eliminated due to these effects.[85]

Lowering the temperature of cyclodextrin columns will result in increased retention and usually increased enantiomeric resolution. At high temperatures, complexation between cyclodextrin and analytes will diminish.[86] This will cause an increase in the relative amount of time an analyte spends in the mobile phase versus the stationary phase.[87]

The polarity of native cyclodextrin CSPs, in the reversed-phase mode, is close to a C_4 or C_8 column. Cyclodextrin-based CSPs often have reduced chromatographic efficiency compared with a typical C_{18} column. The reason is due to the kinetics of association and dissociation with the stationary phase. It is believed that the rate of inclusion complex formation is slower than the rate of mass transfer within the typical brush-type C_4 or C_8 stationary phases. This results in a decreased number of plates per meter at higher flow rates.[84]

2. Normal Phase

In the normal-phase mode of operation, nonpolar organic solvents (hexane, heptane), modified by polar alcohols and ethers, are used as the mobile phase. The nonpolar solvent molecules occupy the cyclodextrin cavity. Consequently, the analytes may only interact with the polar external surface of the cyclodextrin torus and any derivative groups that may be present. Only aromatic-derivatized CSPs have been shown to be effective in the normal-phase mode. There are many types of interactions that, when combined, can give rise to a separation of enantiomers, including hydrogen bonding, steric repulsion, π–π complexation, and dipole–dipole stacking. It seems that the analyte must contain an aromatic group in order to be enantioselectively resolved in the normal-phase mode. This suggests that π–π interaction is the leading interaction for chiral discrimination. As all of the commercially available aromatic derivatized cyclodextrin CSPs are π-electron donating (π-basic), any analyte that is a π-electron acceptor (π-acid) are excellent candidates for enantiomeric separation on these CSPs.[78,88]

The most common mobile phase in normal-phase separation is hexane/isopropanol. Heptane may be used interchangeably with hexane with little change in the separation. Acetone, ethanol, butanol, methyl *t*-butyl ether, and dichloro-methane may be used as polar modifiers. These polar modifiers can contribute to hydrogen bonding or participate in dipolar interactions.[84]

3. Polar Organic Mode

In the polar organic mode, a high percentage of acetonitrile is used as mobile phase. The cyclodextrin cavity is occupied mainly by acetonitrile. This effectively makes the stationary phase more polar, as analytes can only interact with the polar external surfaces (e.g. hydroxyl groups) of the cyclodextrin. The primary interactions that lead to chiral recognition are hydrogen bonding between the secondary hydroxyl groups on

the rim of cyclodextrins and the chiral analyte, dipole–dipole interactions, and steric repulsive interactions. Consequently, chiral analytes must have certain structural features if they are to separate in the polar organic mode. Specifically, an analyte must have a minimum of two separate hydrogen-bonding groups. One of the hydrogen-bonding groups should be on or near the stereogenic center. The other hydrogen-bonding group can be anywhere in the molecule. Also, it is beneficial if the analyte has a bulky moiety near the stereogenic center.[23,89]

The polar organic mode offers some advantages versus the traditional normal-phase mode, which uses hexane or heptane as the main eluent. Most compounds that exist as salt forms (HCl salt, for example) can be dissolved and separated. In addition, eluent from a reversed-phase column can be switched directly onto a chiral column by using a column-switching device.

The typical starting mobile-phase composition is 95:5:0.3:0.2 (by volume) acetonitrile/methanol/AA/TEA. Separation in the polar organic mode may be tuned by changing the relative amounts of the acid/base modifiers. The degree of protonation and charge of the analyte (where applicable) is controlled through the relative amounts of AA and TEA. Optimizing the ratio of amine to acid is essential in optimizing enantioselectivity. To determine the optimal ratio of acid to base modifier the concentration of each modifier is varied while the total volume of modifier is kept constant. Methanol can be added to adjust retention. Since methanol competes with the analyte for hydrogen-bonding sites on the cyclodextrin, it decreases both retention and enantioselectivity.[82,84]

D. π-Complex (Pirkle-Type) CSPs

CSPs that interact with racemic analytes through one or more π–π interactions are called π-complex CSPs. The chiral selector contains either a π-acid or π-base moiety. If the CSP is a π-acid, then the analyte to be resolved needs to contain a π-basic group or vice versa. Other simultaneous interactions have to be present as well if enantioselective separations are to occur. Generally, those interactions include hydrogen bonding, steric repulsion, and dipolar interactions. Separations on the π-complex CSP usually are performed in the normal-phase mode because π–π interactions, hydrogen bonding, and dipolar interactions are more pronounced in nonpolar solvents.

Mikes and Boshart first developed π-acidic and π-basic CSPs for HPLC.[90] Later, Pirkle and coworkers developed analogous CSPs and commercialized some of them.[27,91] Hence, this type of CSP is sometimes referred to as a Pirkle-type CSP. The most recent versions of π-complex CSPs, the (*S*,*S*)-Whelk-O 1 CSP, for example, contain both π-acid and π-base groups, which make them more widely applicable than earlier CSPs.[34]

π-Complex CSPs offer the following advantages: (i) the generally small size of the chiral selector permits a rather dense functionalization of the chromatographic surface, resulting in a CSP with improved column capacity; (ii) both enantiomers of the chiral selector used in many CSPs allow preparation of the CSP in either single enantiomeric form or as a racemic version. This offers highly useful options in trace analysis of enantiomeric purity and assignments of absolute configuration; (iii) incremental changes in the structure of the CSP can be made and the effect on enantioselectivity can be studied. Furthermore, spectroscopic methods can be used to study the causes of enantioselectivity. Consequently, the mechanism of action of this type of CSP is often better understood than that of other CSPs.[92]

1. Method Development

Empirically, the following sequence of columns is recommended to start method development in order of preference: Whelk-O1, ULMO, DACH-DNB, α-Burke, β-Gem, Pirkle 1-J, Leucine, and Phenylglycine. Pirkle-type phases can be used in both the reversed-phase and normal-phase modes, but typically perform best with normal-phase solvents. Typical mobile-phase combinations are as follows: Hexane(heptane)/IPA (ethanol); Hexane(heptane)/CH_2Cl_2/ethanol; Hexane/ethyl acetate; Methanol(ethanol, acetonitrile, THF)/H_2O; Methanol(ethanol)/CH_2Cl_2. It is recommended that a high percentage (~50%) of strong solvent (ethanol, IPA, etc.) be used as a starting point to ensure that all peaks will elute off the column quickly. Mobile-phase modifier (usually ~0.1%) can be added. For basic or amine groups, add TEA, DEA, or ammonium acetate. For acidic groups, add AA, TFA, or ammonium acetate.[93,94]

E. Protein-Based CSPs

In 1973, BSA-agarose was used for the enantioseparation of tryptophan, which was the first reported use of a protein-based CSP for chiral resolution purposes.[92] Since then, various protein-based CSPs have been developed including albumins such as bovine serum albumin (BSA)[95,96] and human serum albumin (HSA);[97] glycoproteins such as AGP;[98] ovomucoid from chicken egg whites (OMCHI), ovoglycoprotein from chicken egg whites (OGCHI), avidin (AVI), and riboflavin binding protein (RfBP) (or flavoprotein); enzymes such as trypsin, α-chymotrypsin, cellobiohydrolase I (CBH I), lysozyme, pepsin, and amyloglucosidase; and other proteins such as ovotransferrin (or conalbumin) and β-lactoglobulin. Protein fragments or protein domains have also been used to prepare CSPs, which is helpful in understanding the chiral recognition site(s) of protein-based CSPs by investigating whether or not independent chiral binding sites exist on each fragment or domain. CSPs based on BSA, HSA, AGP, OMCHI, AVI, CBH I, and pepsin are now commercially available.[99]

The protein can be either physically adsorbed or covalently bonded to silica gel. Aqueous mobile phase is generally used on protein-based CSPs. For bonded columns, physical properties of the base materials, spacer length, and binding method affect enantio-resolution. For adsorbed columns, multilayer adsorption of the protein on the base materials may occur, and the adsorbed protein can be eluted. No matter which approach is used, changes in eluent pH, charged additive, and temperature can induce a conformational change of the protein, which may cause lack of column ruggedness. Protein-based CSPs can attain enantioseparation of a wide range of compounds because of multiple binding interactions and/or multiple binding sites. Hydrophobic, electrostatic, and hydrogen-bonding interactions are responsible for chiral recognition on protein-based CSPs.[99]

Various factors, such as eluent pH, type, and concentration of organic modifier and charged additive, ionic strength and temperature, affect retention and enantioselectivity of the solutes on protein-based columns. Hydrogen-bonding properties and hydrophobicity of the uncharged organic modifier influence enantioselectivity of the solute significantly. Usually, a decreased retention is observed with increased amount of organic modifier in the mobile phase. Separation of enantiomers on a protein-based CSP is generally attained between pH 3 and 8 since a protein sometimes undergoes denaturation in strong acidic or alkaline solution. Furthermore, addition of charged additives such as *N*,*N*-dimethyloctylamine, tetrapropylammonium bromide, tetrabutylammonium bromide, and sparteine affected the retention and enantioselectivity of uncharged, anionic, and cationic solutes. These additives compete with the solute enantiomers at binding site(s).[99]

F. Ligand-Exchange CSPs

LEC was suggested by Helfferich in 1961 and further developed by Davankov into a powerful chiral chromatographic method.[100,101] In 1971, this technique resulted in a complete and reliable separation of enantiomers of racemates that were able to form complexes with transition metal cations.[101] Since then, LEC has been extensively investigated for direct resolution of enantiomers and in mechanism studies.[102]

CSPs in LEC can either be polymers made of chiral ligands, or chromatographic supports (usually silica gel) with chiral ligands adsorbed or chemically bonded on their surface. During the chromatographic process, the metal ion, the chiral ligand, and the analyte molecule form a ternary sorption complex. The reversible conversion of the analyte into the complex happens to be a relatively slow process. It causes a significant resistance to mass transfer in the stationary phase, thus resulting in low efficiency of many chiral ligand-exchanging stationary phases.[26,100]

The following factors were found to influence, in particular, efficiency of bonded chiral LEC phases: flow rate of the mobile phase and structure of the analyte ligand (dentation number, steric shape, bulkiness and rigidity of the molecule, configuration). The pH of the aqueous–organic eluent and the type and concentration of organic modifier are the primary parameters that influence retention of the analytes. Elevated temperature improves the efficiency considerably. Unfortunately, most chiral bonded LEC phases are not hydrolytically stable enough in aqueous and aqueous–organic media at elevated temperatures. Polymeric bonded phases do not have this limitation and can be used at as high as 75°C.[102]

G. Crown Ether CSPs

Crown ether CSPs can be divided into two types according to the chiral units incorporated into chiral crown ethers. The first type is chiral crown ethers incorporating a chiral 1,1′-binaphthyl unit. Bis-(1,1′-binaphthyl)-22-crown-6 was immobilized on silica gel or polystyrene to serve as a CSP for the separation of amino acids and derivatives in the late 1970s.[103] (3,3′-Diphenyl-1,1′-binaphthyl)-20-crown-6 was covalently bonded to silica gel and was applied to separate various α-amino acids, amines, amino alcohols, and related amino compounds.[104] The second type of chiral crown ether CSP is based on the tartaric acid unit, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid.[105] Chiralpak CR is one of the commercial crown ether CSPs that is dynamically coated on octadecyl silica gel.[31]

The anchoring of protonated primary amino groups of chiral compounds inside the cavity of 18-crown-6 ring via three $+N-H\cdots O$ hydrogen bonds is essential for chiral discrimination. Most α-amino acids, primary amines, primary amino alcohols, and racemic drug containing a primary amino group were resolved on common crown ether CSPs. Only a limited number of β-amino acids are resolved.[106]

Organic modifiers in the mobile phase are a very important factor influencing the enantioseparation on crown ether-based CSPs. Water or water–organic solvent mixture containing a small amount of acidic modifier has been used as mobile phase. Methanol and acetonitrile are the most widely used organic modifiers. Ethanol, isopropanol, or tetrahydrofuran can also be used as organic modifier. The effect of organic modifier content in aqueous mobile phase on enantioselectivity is different with different crown ether CSPs and there is no clear trend. The retention behaviors for the resolution of racemic compounds on crown ether-based CSPs are expected to stem from the balance between the lipophilic interaction of analytes with a CSP and the hydrophilic interaction of analytes with mobile phase.[106]

Acidic modifier in the aqueous mobile phase is an important factor influencing the enantioselectivities. Acidic modifiers added to mobile

phase is used to protonate the primary amino group. The protonation improves the complexation of the primary amino group ($R–NH_3^+$) inside the cavity of the crown ether ring of the CSP. Perchloric acid, sulfuric acid, and TFA are the most widely used acidic modifiers. Other acids, such as AA, nitric acid, hydrochloric acid, methanesulfonic acid, and phosphoric acid, can also be used as acidic modifiers.

Some inorganic modifiers can be added to the mobile phase to influence retention. The cation of the inorganic modifier in the mobile phase is expected to compete with the analyte ammonium ion ($R–NH_3^+$) for complexation inside the cavity of the crown ether and consequently reduce retention of the analytes. Ammonium acetate, ammonium chloride, or potassium chloride has been used as inorganic modifier. The retention time decreased as the inorganic modifier concentration increased, but the separation factor and resolution showed maximum values at an appropriate concentration of the inorganic modifier. TEA has been used as a mobile-phase modifier to enhance resolution of α-amino acids. Addition of TEA to aqueous mobile phase increased the retention dramatically and the separation factors improved with increasing TEA.[106]

Column temperature is another important factor influencing the resolution of primary amino compounds. Lower temperature will enhance the retention and separation factor. At lower temperature, the diastereomeric complexes formed between the individual enantiomers of an analyte and the chiral crown ether moiety is expected to become energetically more favorable.[106]

H. Synthetic Polymer/Imprinted Polymer CSPs

The principal synthetic polymers used as CSP are poly(triphenylmethylmethacrylate) and poly(2-pyridyldiphenylmethylmethacrylate).[107] Most compounds resolved on these CSPs are better resolved on other CSPs.

Among synthetic polymers, MIPs are tailored for the separation of target chiral molecules. MIPs can be prepared according to a number of approaches that are different in the way the template is linked to the functional monomer and subsequently to the polymeric binding sites. Thus, the template can be linked and subsequently recognized by virtually any combination of cleavable covalent bonds, metal–ion coordination, or noncovalent bonds.

The most widely applied technique is represented by the noncovalent route developed by Mosbach et al.[108] The most successful noncovalent imprinting systems are based on commodity acrylic or methacrylic monomers, such as methacrylic acid, cross-linked with ethyleneglycol dimethyacrylate.

In the first step, the template, the functional monomer, and crosslinking monomer are dissolved in a poor hydrogen-bonding solvent of low to medium polarity. Free radical polymerization is then initiated

with an azo initiator by photochemical homolysis or thermochemically at 60°C or higher. In the final step, the resultant polymer is crushed by mortar and pestle, extracted using a Soxhlet apparatus and sieved to a suitable particle size for chromatographic applications.

The advantage of using enantiomers as templates is that nonspecific binding, which affects both enantiomers equally, cancels out. Therefore, the separation factor uniquely reflects the contribution to binding from the enantioselectively imprinted sites. Efficiency, resolution, and selectivity are affected by the quality of the packing and mass transfer limitations as well as by the amount and distribution of the binding sites.

Some restrictions of the molecular imprinting techniques are obvious. The template must be available in preparative amounts, it must be soluble in the monomer mixture and it must be stable and unreactive under the conditions of the polymerization. The solvent must be chosen based on stability of the monomer–template assemblies and whether it results in the porous structure necessary for rapid kinetics in the interaction of the template with the binding sites.

A large number of racemates have been successfully resolved on the tailor-made molecularly imprinted CSPs. Using MAA as functional monomer, good recognition is obtained for templates containing Bronsted-basic or hydrogen-bonding functional groups close to the stereogenic center. In contrast, templates containing acidic functional groups are better imprinted using a basic functional monomer such as vinylpyridine. This emphasizes that it is important to use complementary functional groups when designing the CSPs. Furthermore, the separation factors are much higher than those observed on many commercial CSPs. However, each column can only resolve a limited number of racemates and the resolution is generally low. At low sample loads, the retention on MIP CSPs is extremely sensitive to the amount of sample injected because of overloading of the small number of high-energy binding sites. The peaks are usually broad and asymmetric due to the site heterogeneity and slow mass transfer.

For low to moderately polar templates, good recognition is generally seen using organic solvents in a rebinding media where the template interacts mainly electrostatically with the binding sites. In fact, most rebinding experiments to MIPs demonstrating high affinity and selectivity have been performed using organic solvents. Therefore, the first chromatographic rebinding evaluation for most templates is often carried out in organic mobile-phase systems.

I. Other Types of CSPs

In addition to the CSPs discussed above, other types of CSPs have been explored as well. Some of them exhibit interesting resolution capability for enantiomers. CSPs possessing charged functional groups act as

ion-exchangers if the chiral analyte to be separated possesses oppositely charged functional groups. These CSPs are especially useful for the separation of very polar charged analytes, such as sulfonic acids. Chiral anion exchangers, such as those derived from chincona alkaloids quinine and quinidine, are successful examples.[109,110] A more comprehensive review of new CSPs is provided in reference 111. Many new CSPs are still in the research stage and not commercialized and currently do not have a significant impact on the pharmaceutical industry.

J. Summary

To choose the right column for method development for chiral pharmaceutical analytes, many factors should be considered, which include, for example, structural characteristics of the analyte; solubility of the sample in the mobile phase; cost of the column; mobile phase and waste treatment; analysis time; method robustness or transferability; and the need for conversion of the analytical method to a preparative method, etc. For the majority of analytical-scale chiral separations, the two universal types of CSPs (polysaccharide and macrocyclic glycopeptide) will provide sufficient enantio-resolution. Among the universal CSPs, Chialpak AD-H, Chiralcel OD-H, Chirobiotic T, and Chirobiotic V offer the broadest selectivity. Choice of the polar organic, reversed-phase, or normal-phase mode as a starting point depends on the characteristics of the analyte. To increase the success rate of trial-and-error approach, individual experience/expertise should be combined with other approaches such as use of the databases and column scouting.

III. PRACTICAL GUIDELINES TO CHIRAL HPLC OF PHARMACEUTICALS

A. Column Selection Aided by Chiral Database

Achievement of a chiral separation often requires large numbers of expensive and time-consuming laboratory experiments, even for experienced chemists. The trial-and-error approach may result in loss of time and resources by repeating negative experiments already mentioned in the literature. Substantial savings in resources can be expected by use of a chiral database searchable based on molecular structures. CHIRBASE, a molecular-orientated factual database was built by Roussel and Piras in 1989.[112] The database includes several tens of thousands of entries and the entire system can be searched by the ISIS software. The purpose of the database is to gather together comprehensive structural, experimental, and bibliographic information on successful and unsuccessful chiral separations that have been obtained on CSPs by liquid chromatography.[113]

Many of the major column manufacturers offer practical background information on their columns as well as technical assistance for those interested in developing analytical and/or preparative enantiomeric separations. For example, Astec, Daicel (Chiral Technologies in the United States and Europe), Regis Technologies, and ChromTech AB have accumulated substantial libraries of LC enantiomeric separations. They have handbooks and/or tables of separation data along with information on the use and care of their columns. Much of this information is available on their respective Websites. The material is usually current and often contains the latest strategies for obtaining good enantiomeric separations. In addition, the major manufacturers will develop customized analytical and/or preparative separations for interested customers, and they will contract to do the separations in-house, if the customer prefers.

Some research organizations, small analytical groups or individuals have attempted to build private databases by accumulating chiral separation data on proprietary compounds. In combination with the commercial and vendor database, these private databases provide very practical and useful information for method development of proprietary compounds.

B. Screening Approach

The common stepwise trial-and-error approach relies on the analyst's experience and understanding of the enantiorecognition mechanism between the chiral selector and the analyte. Even with the help of literature or databases, this approach can be time consuming. It is not always efficient in the pharmaceutical industry where methods need to be developed for a large number of compounds.

With over 170 CSPs commercially available, reliable and rapid enantioselectivity prediction of a new chiral molecule with any CSP is not currently available. Small changes in structure and/or chromatographic environment have shown great impact on the ability to resolve a racemate for many CSPs. Consequently, screening approaches emerged, which are able to analyze quickly a series of molecules with very diverse structure and chemical properties with a minimum set of experimental conditions. The key point for developing rapid screening schemes is to include a reduced set of CSPs with broad application range. Of the commercially available CSPs, the polysaccharides and macrocyclic glycopeptides have appeared to be broad-spectrum and have been chosen by various groups for the screening strategies in HPLC.

Currently, there are three automated column-screening approaches: (i) automated column switching in sequence; (ii) screening using column coupling; and (iii) multicolumn parallel screening. For example, four polysaccharide-based CSPs and three macrocyclic glycopeptide-based

CSPs were evaluated for rapid HPLC screening for the separation of over 55 chiral compounds of pharmaceutical interest.[114] The polysaccharide columns were employed in the normal-phase and polar organic modes and showed overall enantioselectivity for 87% of the compounds tested. The macrocyclic glycopeptide columns were employed in the reversed-phase and polar organic mode and showed enantioselectivity for 65% of the analytes. When the results from both the polysaccharide and the macrocyclic glycopeptide screen were combined, they showed enantioselectivity for 53 out of 55 enantiomeric pairs (96%). This leads to the conclusion that the two screens have complementary properties, i.e. both types of columns should be included to achieve the highest probability of success. The screens were automated using a column switcher that allowed for different combination of CSP and mobile phase to be tested overnight. The method development can be achieved within 24 h for each compound.

The column coupling approach was applied on macrocyclic glycopeptide CSPs. Although the macrocyclic glycopeptides have analogous structures, they have somewhat different enantioselectivities. One feature of this class of chiral selectors is their "principle of complementary separations". This means that if a partial enantiomeric separation is obtained with one glycopeptide, there is a strong probability that a baseline or better separation can be obtained with a related Chirobiotic CSP using the same or a similar mobile phase. The reason for this phenomenon is the subtle differences in the stereoselective binding sites between these related CSPs.

Since each of the chirobiotic columns has its unique selectivity in all three mobile-phase modes toward different chiral racemates, it is a very efficient way to put them together to do a fast screening for selectivity. The coupled column consists of three 10 cm columns in the order of increasing polarity regardless of the mobile-phase type, i.e. Chirobiotic R, V, and T. This technique allows evaluation of this entire class of chiral selectors with a single coupled column for the ability to separate a molecule. Even if a partial separation is obtained on the coupled column, a baseline separation is guaranteed with one of the columns in this class.[115]

The parallel multi-column screening approach was published by Zhang et al.[25] The modified HPLC system allows simultaneous screening of five CSPs in parallel using a regular HPLC auto sampler and a pump with five UV detectors.

IV. CONCLUSIONS

The importance of chiral separation in the pharmaceutical industry is well recognized and emphasized by regulatory authorities. The separation

techniques are dominantly based on HPLC that provides reliable and convenient methods. Column selection is the most important and challenging step of method development. When choosing a column, in addition to its selectivity, the mode of operation, compatibility of mobile phase with sample solvent, robustness, efficiency, loadability, and reproducibility should be considered.

Thanks to the rapid development of HPLC CSPs in the past few decades, the majority of chiral separations can be achieved on two types of universal columns, polysaccharide-based CSPs and macrocyclic glycopeptide CSPs. Both types of column can be operated in the reversed-phase, normal-phase and polar organic modes with complementary enantioselectivities. This makes it possible for a method developer who is new to the chiral separation field to gain expertise within a short time. Chiral databases of various origins, as well as the column screening approach, help improve efficiency for developing an effective chiral separation method. For the more difficult separation problems, a broader knowledge base and/or more experience are needed. It is still difficult, if not impossible, to predict a separation of a new chiral molecule. This difficulty has been and will continue to be the driving force for development of break-through technologies for chiral separations.

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6

CONTEMPORARY LIQUID CHROMATOGRAPHIC SYSTEMS FOR METHOD DEVELOPMENT

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ABSTRACT

There are several basic components to a liquid chromatographic (LC) system used for method development, and modern technology, from column chemistry to advanced detection and automation, is quickly changing the landscape of method development. The way in which solvents and samples are managed, and the types of columns and detectors used can

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have a significant impact on both the throughput (time it takes to develop a method) and the quality and/or robustness of the method.

This chapter will begin with a brief description of a basic high performance liquid chromatography (HPLC) method development system, and then discuss in some detail modern ultra performance liquid chromatography (UPLC™) instrumentation components and how they are used to develop methods for the types of complex samples encountered in today's pharmaceutical laboratory. Recent advances in UPLC technology, capitalizing on sub-2 μm particle column packings, automated approaches, and migrating from HPLC to UPLC will also be addressed.

I. INTRODUCTION

There are many different approaches to method development used in LC, from simple "trial-and-error", to more complex multivariate approaches involving experimental design and/or chemometrics. Regardless of the approach, however, most of the basic instrument components remain the same; the solvent manager (pump), the sample manager (injector), the column and column module, the detector, and some means of recording the results; these days this is most likely to be a chromatography data system (CDS). For the most part, LC systems and components have remained unchanged for at least the last 30 years. New detectors have been introduced, and refinements made, but until recently, any LC method in use could be run on systems of early 70's vintage. In 2004, the first LC system capable of operation up to 15,000 psi (1000 bar) was introduced,[1–6] one of the first true advancement of LC technology in three decades. The combination of a system capable of high pressure operation and columns packed with sub-2 μm particles has been termed ultra performance liquid chromatography (UPLC™) to differentiate it from HPLC.[3]

Improvements in resolving power (1.7×), sensitivity (3×) and separation speed (9×) were demonstrated for many different applications.[3,4] All these benefits are derived from the 1.7 μm particles used in UPLC columns.

Since over the years many excellent reviews have appeared describing LC instrumentation in great detail (e.g. References 7–10), it is not the intent of this chapter to reinvent the wheel. Therefore for more in depth information on traditional LC systems the reader is encouraged to consult the literature referenced here and elsewhere. Instead, this chapter will look at how LC technology has evolved over the years relative to method development, but more importantly focus on recent trends aimed at improving speed, sensitivity, and resolution while developing high quality, robust methods for the pharmaceutical industry. The use of automation, and modern instrumentation technology designed to take full advantage of the evolution of even smaller particle size column packings (below 2 μm) will also be described.

II. TRADITIONAL INSTRUMENTATION FOR HPLC METHOD DEVELOPMENT

A. System Basics

There are several key components of any HPLC system, and systems used for method development are really no different. HPLC systems can be modular or integrated, and use either isocratic or gradient solvent delivery. Modular systems, as the term implies, consist of separate modules connected in such a way as to function as a single unit. Modular systems can provide a degree of flexibility to exchange different components in and out of the system, sometimes necessary for maintenance purposes or experimental requirements. In regulated laboratories this flexibility may not be viewed as an advantage however due to compliance issues with instrument validation/qualification. In integrated systems, the individual components can share electrical, communication and fluid connections and control, and can operate in ways that provide better solvent and sample management than modular systems. Modern integrated systems are holistically designed to take advantage of managing both the sample and the solvent in ways that can significantly decrease injection cycle time and provide increased precision and accuracy while still providing flexibility in detection choices.

In general, gradient systems are preferred over isocratic systems for method development because of their multi-solvent capability. Gradient multi-solvent systems can be used to prepare mobile phases on the fly, often referred to as "dial-a-mix" or "auto blend", providing maximum flexibility for method development (especially for method and column screening approaches) and the mobile phases are often more robust and accurate than premixed mobile phases when methods are in routine use. Figure 1 illustrates this capability. Figure 1A shows three overlaid chromatographic results from one system, three different chemists, on three different days, using premixed solvents. The chemist-to-chemist reproducibility is seen to be quite variable. In Figure 1B, every tenth injection of 100 runs from an experiment using premixed solvents are overlaid. Figure 1B illustrates that even on a single system, with a single chemist, premixing solvents can affect repeatability over time. Variability in this instance most likely arises from selective evaporative loss of the organic solvent, as later runs have longer retention times. Finally, in Figure 1C, overlaid results are presented for every tenth injection of 100 runs using auto blend, or dial-a-mix; that is, using the system to make the mobile phase. As illustrated, the system is far more accurate in preparing the mobile phases than either a single or multiple analysts premixing the mobile phase. Using auto blend, different organic solvent proportions, buffer strength, and pH can be generated by using the solvent manager to proportionately mix the appropriate stock solutions to obtain the final

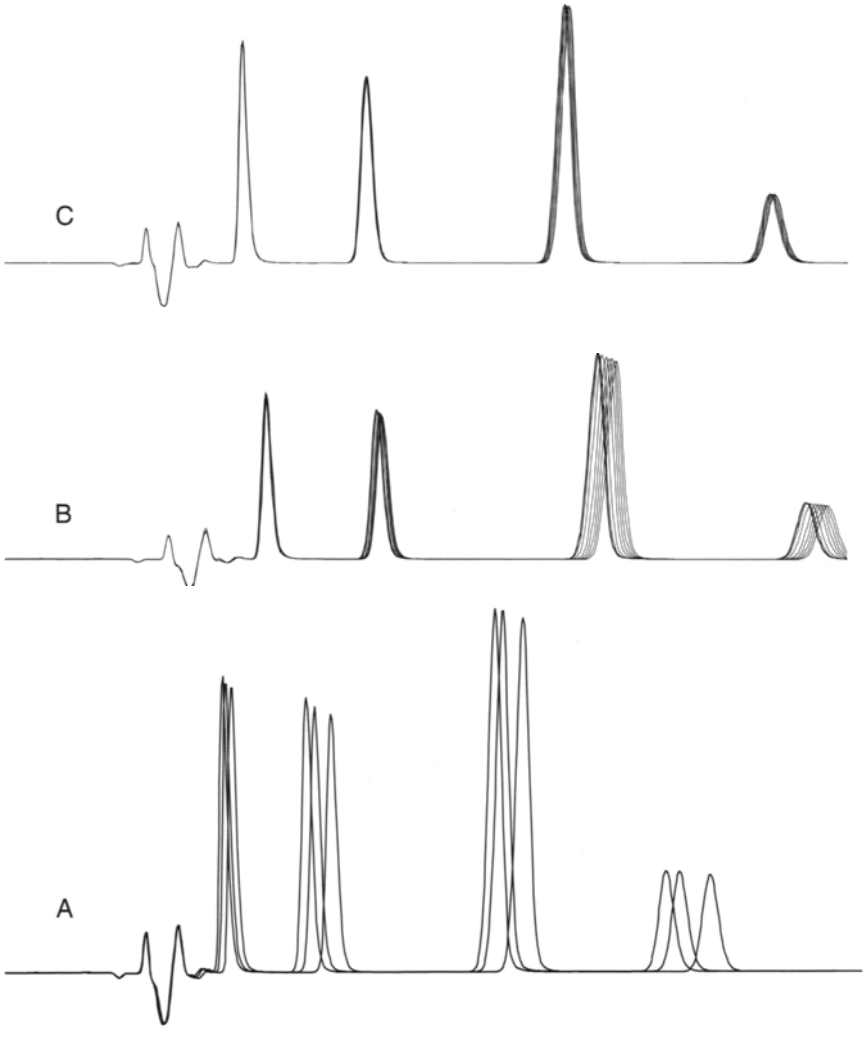

FIGURE 1 **Comparison of premixing mobile phase solvents to auto blend. (A): Three overlaid chromatographic results from one system, three different chemists, on three different days, using premixed solvents. (B): Every tenth injection of 100 runs from an experiment using premixed solvents are overlaid. (C): Overlaid results are presented for every tenth injection of 100 runs using auto blend, or dial-a-mix; that is, using the system to make the mobile phase.**

mobile phase conditions. Auto blending of this type can be used to the analysts' advantage during method development.

The automated blending of solvents might at first seem to be a trivial matter. However, automated method development systems depend on precise and reproducible blending in scouting experiments designed to

study the effects of different mobile phase conditions on selectivity. In the strictest sense, gradient chromatography is essentially auto blending, albeit over time. Isocratic conditions, if desired for the final method, can be determined from gradient conditions, and of course still be run on the gradient system. The kinds of results obtained in Figure 1C are critical to the type of separations illustrated in Figure 2. Figure 2 shows six overlaid chromatograms of a method requiring critical resolution of a series of minor components. Without accurate and precise mobile phase generation and solvent delivery this critical resolution could not be maintained.

B. HPLC Systems for Column and Method Scouting

Method and column scouting is a method development approach commonly used to investigate potential starting conditions for further method optimization. A typical HPLC system used to generate the kind of results obtained in Figures 1 and 2 and run scouting experiments is shown in Figure 3. Most major LC manufacturers systems can be similarly configured into a resulting method development workhorse system. In addition to the basic solvent and sample manager, the system is configured with a solvent and column switching valve, a column oven, and multiple detector capabilities including both photodiode array (PDA) and mass spectrometry (MS). For the most part, PDA and single quadrupole MS are the most useful detectors for method development, and are covered in more detail later in this chapter. As configured, the system is capable of delivering mobile phases consisting of different blends of multiple organic solvents, multiple buffers and pH's, and four or more columns operated at different temperatures. This multi-parameter mobile phase capability provides access to all of the potential variables the analyst needs to investigate when manipulating selectivity in method development.

Scouting or screening systems are often used to identify the most promising conditions (solvent, column pH, etc.) for further optimization or development. Templates are written in the CDS using instrument methods to generate the various mobile phase conditions, equilibrate and/or switch the column, perform all of the chromatographic runs, and run wash or shutdown procedures for both the columns and the system. These templates are usually written once, and then used repeatedly as new methods need to be developed, allowing the analyst to run the system in a semi-automated manner. A typical screening experiment might generate in excess of 16 chromatograms; for example using four different columns, high and low pH, and two different solvents (e.g. methanol and acetonitrile). Of course, multiple additional conditions generate a great deal more chromatographic data, and once generated, all of the data must be scrutinized. However, rather than go through each

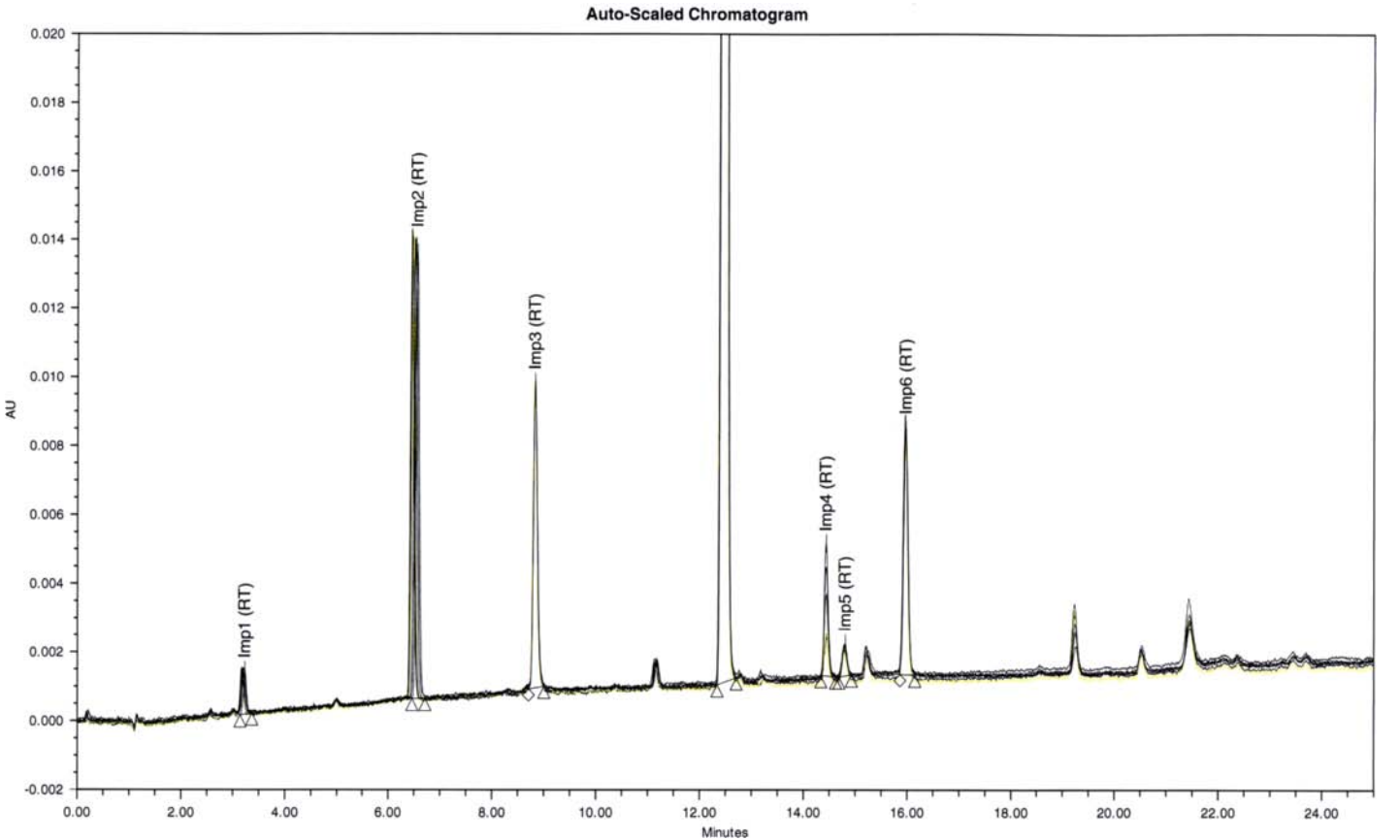

FIGURE 2 Six overlaid chromatograms of a method requiring critical resolution of a series of minor components requiring accurate and precise mobile phase delivery.

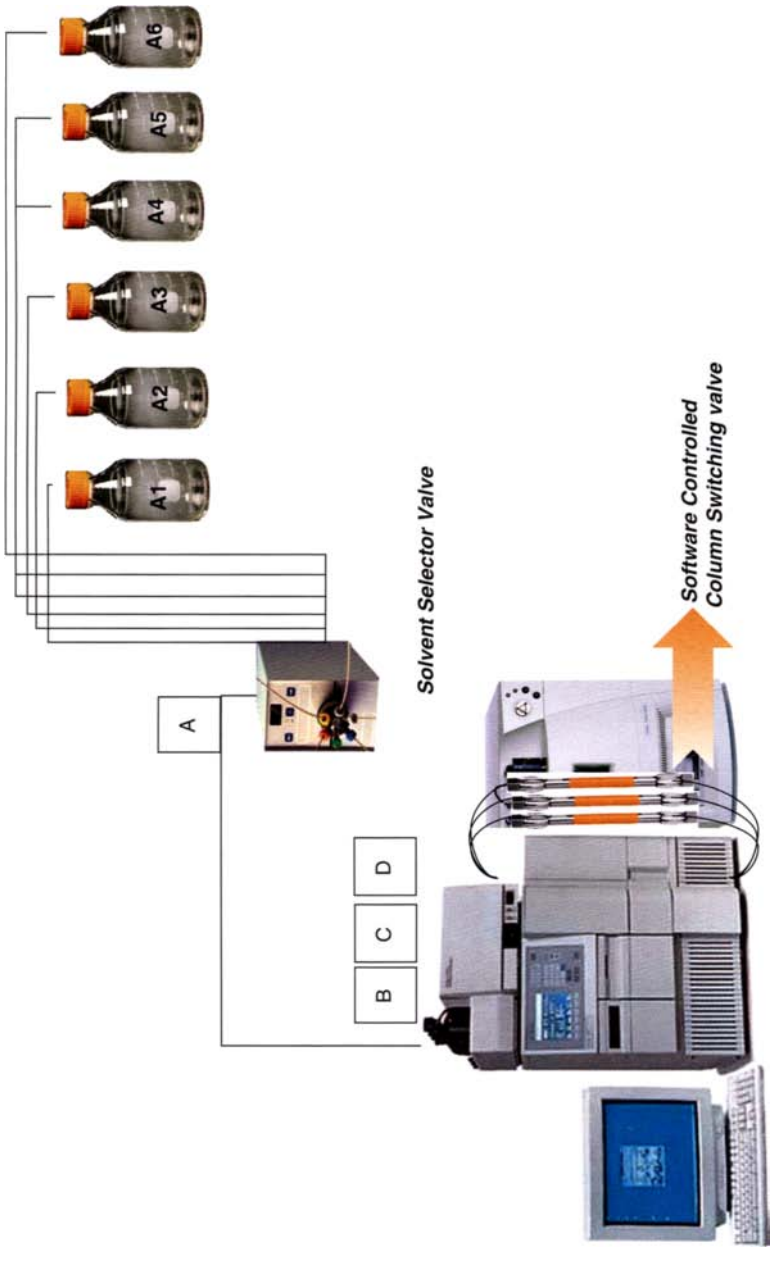

FIGURE 3 Example of a typical HPLC system configured for method development.

chromatogram individually, the analyst can use the custom reporting features of the CDS relational database to generate summary plots that at a glance reveal which runs gave the greatest number of peaks, most resolution, etc.

C. Automated HPLC Method Development Systems

While scouting systems are a step in the right direction, they leave out the critical last step of method optimization. The final automation of the optimization is a natural extension of a methodical, planned method development process. But why automate? The desire to automate method development stems from the simple reason that traditional manual HPLC method development is a labor intensive, time consuming, and often-imprecise process, resulting in lost time, money, and productivity, since it can take weeks or longer to develop a method manually. Automated method development systems provide an alternative to the traditional slow, manual, and unreliable trial-and-error method development approach and can often reduce method development time to as little as a few hours.

Recently, systems have been developed that utilize external modeling software (e.g. DryLab®, Rheodyne Inc., Rohnert Park, CA and LC Simulator or AutoChrom, Advanced Chemistry Development, Toronto), which either partially or completely automate the rest of the HPLC method development process.

Using theoretical based modeling software allows analysts to evaluate a much wider range of experimental conditions than would ever be practical by running experiments in the laboratory, significantly decreasing method development time. With this type of software, the effects of variables like the organic concentration, pH, temperature, and buffer concentration can be easily observed. In addition, analysts can:

- Evaluate method robustness to decrease the cost of revalidating methods.
- Transfer gradient methods from one instrument to another, eliminating method redevelopment time.
- Model two separation variables simultaneously for faster method development.
- Shorten run times to increase sample throughput.
- Train new chromatographers and establish laboratory method development SOP's.

The critical component to a completely automated system is software that can make decisions from both real and predictive data; software that bridges the gap between the modeling software and CDS software that runs the system and generates data. In these systems the process of method

development starts with the help of a Windows-based wizard, an interface between the modeling software (e.g. DryLab) and the CDS. The wizard asks for specific information about separation needs, and using software protocols suggests actual starting conditions, including pH, solvent, and column. The wizard also facilitates the setup of the method in the CDS and the system completes the analyses. A PDA spectral-based peak-tracking algorithm allows more accurate identification of sample components during the method development process, identifying peaks as selectivity and therefore elution order changes over the course of a few "chemistry calibration" runs. Once the calibration runs are processed the chromatography variables are quickly modeled, and an optimized chromatographic method prediction is obtained. Using systems of this type, with intelligent decision-making software, it is not uncommon to optimize a method in as little as four or five chromatographic runs over just a few hours.[11,12]

III. CONTEMPORARY METHOD DEVELOPMENT SYSTEMS

A. Taking Advantage of Decreasing Column Particle Sizes

One of the primary drivers for the growth and continued use of HPLC has been the evolution of packing materials used to effect the separation. The underlying principles of this evolution are governed by the van Deemter equation:

$$H = \mathrm{A}(d_p) + \mathrm{B}/u + \mathrm{C}(d_p)^2 u$$

which describes the relationship between H, plate height (HETP or column efficiency), linear velocity, u, (flow rate) and particle size or diameter, d_p.

The "A" term represents eddy diffusion, the "B" term represents longitudinal diffusion, and the "C" term represents resistance to mass transfer in and out of the particle.

Since particle size is one of the variables, a van Deemter curve can be used to investigate chromatographic performance.

According to the van Deemter equation, as the particle size decreases to less than 2 μm, not only is there a significant gain in efficiency; but the efficiency does not diminish at increased flow rates or linear velocities.[13] By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits, termed UPLC™. UPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles, and/or higher flow rates for increased speed, with superior resolution and sensitivity.

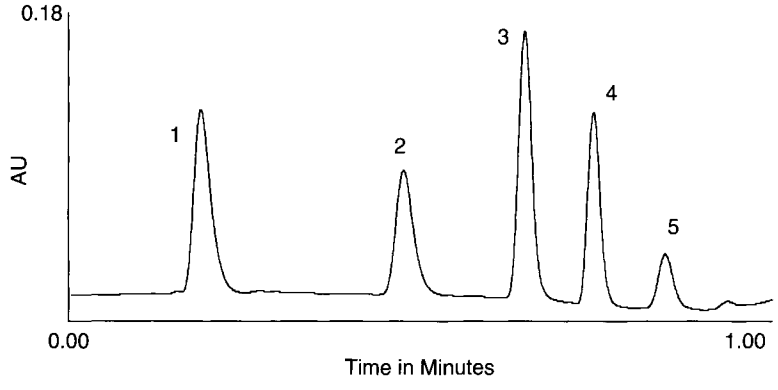

FIGURE 4 UPLC stability indicating assay. UPLC conditions: column: 2.1 by 30 mm 1.7 µm ACQUITY BEH C18 at 30°C. A 45 s 5–85%B linear gradient, at a flow rate of 0.8 mL/min was used. Mobile phase A was 10 mm ammonium formate, pH 4.0, B was acetonitrile. UV detection at 273 nm and 40 pts/s. Peaks are in order: 5-nitroso-2,4,6-triaminopyrimidine, 4-amino-6-chloro-1, 3-benzenesulfanamide, hydrochlorthiazide, triamterine, and methylbenzenesulfanamide; 5 µL injection, 0.1 mg/mL each.

Figure 4 shows a stability indicating assay of five related substances accomplished in under one minute, proving that the resolving power of UPLC is not compromised even at high speed. The current USP lists multiple HPLC methods for the analysis of these same compounds with run times approaching 20 min, with broad, tailed peaks. With such short, high resolution runs, UPLC can result in significant time savings in the method development laboratory.

B. Chemistry of Small Particles

Smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as HPLC. In the fundamental resolution (Rs) equation:

$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{k+1}\right)$$

resolution is proportional to the square root of N. But since N is inversely proportional to particle size (d_p):

$$N \propto \frac{1}{d_p}$$

as the particle size is lowered by a factor of 3, say from 5 μm (HPLC-scale) to 1.7 μm (UPLC-scale), N is increased by 3 and resolution by the square root of 3 or 1.7. N is also inversely proportional to the square of the peak width:

$$N \propto \frac{1}{w^2}$$

illustrating that the narrower the peaks are, the easier they are to separate from each other. Also, peak height is inversely proportional to the peak width:

$$H \propto \frac{1}{w}$$

so as the particle size decreases to increase N and subsequently Rs, an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient separations, desirable for many applications, e.g. peptide maps.

Still another equation comes into play when migrating toward smaller particles:

$$F_{\text{opt}} \propto \frac{1}{d_p}$$

A relationship also revealed from the van Deemter plot. As particle size decreases, the optimum flow to reach maximum N increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressures, and a system properly designed to capitalize on the efficiency gains; a system that can both reliably deliver the requisite pressures and that can maintain the separation efficiency of the small particles with tightly managed volumes.

Higher resolution and efficiency can be leveraged even further, however, when analysis speed is the primary objective. Efficiency is proportional to column length and inversely proportional to the particle size:

$$N \propto \frac{L}{d_p}$$

Therefore the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to the smaller particles and shortening the column by one third (again due to the smaller particles) the separation is complete in 1/9 the time while maintaining resolution. So if speed, throughput, or sample capacity is a concern, theory can be further leveraged to get much higher throughput.

But the design and development of sub-2 μm particles is a significant challenge, and researchers have been active in this area for some time to

try and capitalize on their advantages.[5,13,14] Although high efficiency, non-porous 1.5 μm particles are commercially available, they suffer from poor loading capacity and retention due to low surface area. Silica based particles have good mechanical strength, but can suffer from a number of disadvantages, which include a limited pH range and tailing of basic analytes. Polymeric columns can overcome pH limitations, but have their own issues, including low efficiencies, limited loading capacities, and poor mechanical strength.

In 2000, Waters introduced XTerra™, a first generation hybrid-chemistry that took advantage of the best of both the silica and polymeric column worlds. XTerra™ columns are mechanically strong, with high efficiency, and operate over an extended pH range. They are produced using a classical sol–gel synthesis that incorporates carbon in the form of methyl groups. But in order to provide the necessary mechanical stability for UPLC, a second generation bridged ethane hybrid (BEH) technology was developed. Called ACQUITY UPLC™ BEH, these 1.7 μm particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix.

Particle size distribution is also important in maintaining the high efficiency that theory suggests. Theory would be perfectly supported if every particle in the column was 1.7 μm. However, due to synthesis and sizing limitations, that is not possible. Instead, the narrowest particle size distribution provides the greatest efficiency; wider or skewed distributions dilute or decrease efficiency (contributions from larger particles) or provide abnormally high back pressures (contributions from smaller particles).

Packing 1.7 μm particles into reproducible and rugged columns was also a challenge that needed to be overcome. Requirements include a smoother interior surface of the column hardware, and re-designing the end frits to retain the small particles and resist clogging. Packed bed uniformity is also critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations.

C. Capitalizing on Smaller Particles

Instrument technology also had to keep pace to truly take advantage of the increased speed, superior resolution, and sensitivity afforded by smaller particles. Standard HPLC technology simply does not have the capability to take full advantage of sub-2 μm particles. Larger system volume, larger bandspread, and a limited pressure range limit HPLC's usefulness when using sub-2 μm particles. A completely new system design with advanced technology in the solvent and sample manager, auto sampler, detector, data system, and service diagnostics is required. UPLC systems are holistically designed for low system and dwell volume to minimize dispersion and take full advantage of small particle technology. Figure 5 shows an example of a commercially available UPLC instrument, the Waters ACQUITY UPLC™ system.

FIGURE 5 Waters ACQUITY UPLC™ system.

1. Solvent Management

LC pumps are sometimes categorized according to the way solvents are blended. Low pressure designs use a single pump to deliver mobile phases generated by an upstream proportioning valve. High pressure systems use two or more pumps to proportion solvents downstream at high pressure. As illustrated in Figure 6, a significant difference between high pressure and low pressure systems is in the system volume. While low pressure systems usually exhibit less compositional ripple in the chromatographic baseline, high pressure systems usually have lower volumes. Therefore, if speed or high throughput is desired, high pressure systems are preferred.

It is important to remember that a proper determination of the volume is important for any system used in method development. Problems related to method transfer can often be traced to differences in system, dwell, or gradient delay volumes, since no two systems will have exactly the same volume. The volume difference is particularly significant when transferring methods between low pressure and high pressure systems. In addition, problems may also result from how the volumes are calculated.[15] Accurate volume determinations for high pressure systems can be made using a step gradient method, because the mobile phase is generated post-pump. For accurate low pressure system volume determinations, a linear gradient must be used, to take into account the pre-pump volume from the solvent proportioning valve. It is for this reason that

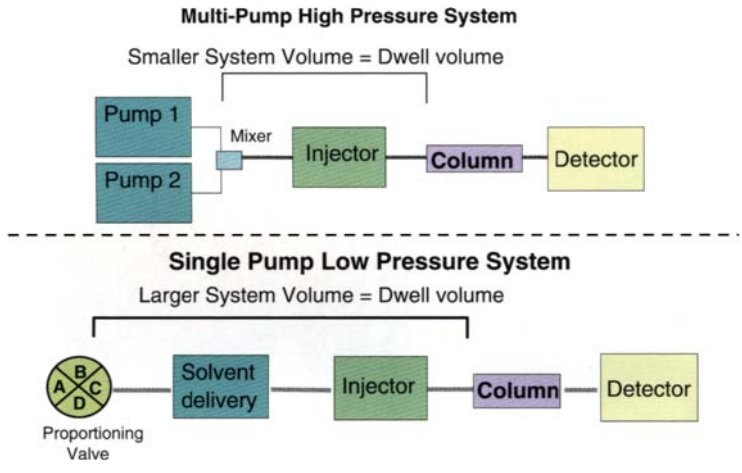

FIGURE 6 **Schematic diagrams of a low pressure mixing system using a single pump and a four position solvent proportioning valve, and a high pressure mixing system using multiple pumps.**

many method developers now recommend programming in a short isocratic step at the beginning of every gradient to accommodate transfer to systems with differing volumes.[16]

Achieving small particle high peak capacity separations requires a greater pressure range than that achievable by traditional HPLC instrumentation. The calculated pressure drop at the optimum flow rate for maximum efficiency across a 15 cm long column packed with 1.7 μm particles is approximately 15,000 psi. Therefore, a pump capable of delivering solvent smoothly and reproducibly at these pressures and that can compensate for solvent compressibility, while operating in both the gradient and isocratic separation modes is required.

In the ACQUITY UPLC system a binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure, as depicted schematically in Figure 7. Each binary solvent manager consists of two independent pump systems, A (left) and B (right), and each independent pump system contains two linear-drive actuators (left and right). Each left and right actuator pair contributes equally (in "parallel") to deliver precise flow of a single solvent.

"First in, first out" serial flow is obtained according to the following order of events:

1. Solvent is degassed by the inline vacuum degasser.
2. The solvents (A1 or A2 and B1 or B2) flow through the inlet check valve into the primary piston chamber while the accumulator piston delivers solvent under pressure to the system pressure transducer.

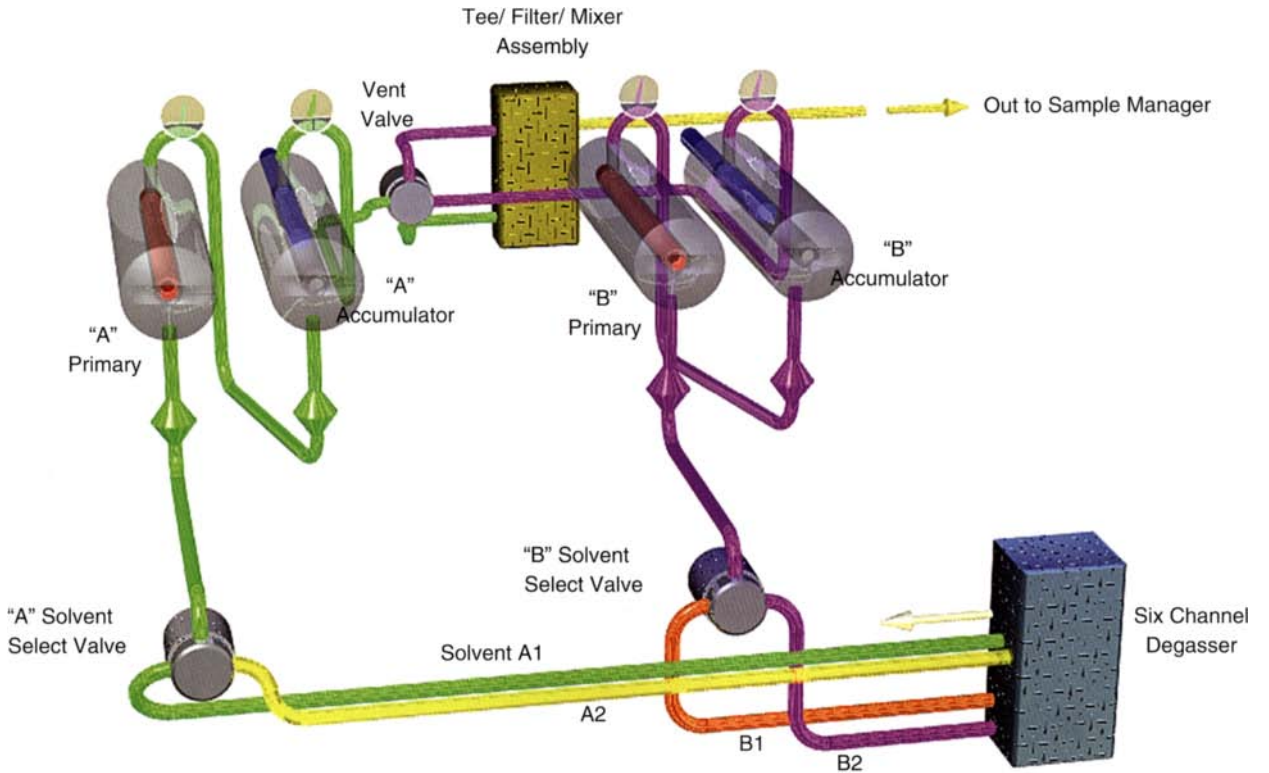

FIGURE 7 Flow path schematic of the Waters ACQUITY UPLC system.

3. Just before the accumulator chamber empties, the solvent in the primary piston chamber is pre-compressed to a pressure slightly less than that indicated by the system pressure transducer.
4. When the accumulator piston chamber is empty, the primary piston delivers solvent under pressure through the primary pressure transducer, refills the accumulator piston chamber, and delivers solvent under pressure through the system pressure transducer, while maintaining a constant flow through the system. The cycle repeats, beginning at step 2.
5. The system pressure transducer measures the operating pressure. A software algorithm compares the primary head pressure and the system pressure readings and regulates the pre-compression step to balance the pressures, providing a smooth, ripple-free flow.
6. Flow continues from the system pressure transducer outlet to the vent valve and into a filter/mixer/tee assembly where the solvents are mixed.
7. The solvent flow proceeds to the sample manager.

The constant and precise solvent delivery satisfies the requirements to take maximum advantage of sub-2 μm particles, as well as the automated generation of mobile phases (autoblend) for modern method development approaches.

2. Sample Management

Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure, and to protect the column from experiencing extreme pressure fluctuations, the injection process must be relatively pulse-free. The swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to realize the increased sensitivity benefits. Low dispersion in the UPLC system is maintained through the injection process using pressure assist sample introduction, and a series of pressure transducers facilitate self monitoring and diagnostics. Injection cycle time is 25 s without a wash and 60 s with a dual wash used to further decrease carry over. A variety of microtiter plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional sample organizer, the sample manager can inject from samples from up to 22 microtiter plates.

(a) Column Module:

Running a column at room temperature, or even "controlled" room temperature, is a thing of the past. Requirements for accuracy and precision require that the column be thermostatted. Since temperature can also be used as a selectivity tool, modern method development systems require

a column heater module. In the ACQUITY UPLC system, the sample manager also controls the column heater module. Column temperatures up to 65°C can be attained, and varied from the CDS for method development. A "pivot out" design provides versatility to allow the column outlet to be placed in closer proximity to, for example, the source inlet of an MS detector to minimize excess tubing and sample dispersion. An optional version of the column module handles up to four columns and a switching valve to further accommodate method development.

All AQUITY columns also include eCord™ microchip technology that captures the manufacturing information for each column, including the quality control tests and certificates of analysis. When used in the Waters ACQUITY UPLC system, the eCord and the eCord database is updated with real time method information, such as the number of injections, or pressure information, to maintain a complete column history, that is permanently archived. For method developers, this is invaluable information because at a glance, analysts can get a complete column history and determine whether the column is still suitable for use.

3. Detection

(a) PhotoDiode Array (PDA) Detection:

Photodiode array (PDA) detection is commonly used in the laboratory to determine peak identity and purity/homogeneity. Modern detector hardware that achieves 1.2 nm resolution can yield spectral fine structure that, when analyzed by mathematical software algorithms, can reveal spectral differences between compounds with similar structures. Spectral information of this type can be used to track peaks and look for co-elution during method development as selectivity is manipulated, and identify related compounds in impurity profiles; while at the same time still being used for routine single wavelength quantitative analyses.

With the advent of high speed UPLC techniques, new detector technology had to be developed to keep pace. Traditional PDA detectors have suffered from a lack of sensitivity, and an increase in bandspread that resulted in a loss of resolution. A new detector cell that did not contribute to system bandspread and much faster data rates were required to take full advantage of PDA detection in UPLC.

(i) Flow Cell Technology. Conventional absorbance-based optical detectors are concentration sensitive detectors. Since small bore high capacity ACQUITY UPLC columns produce small volume peaks, the UPLC detector flow cell volume must be correspondingly low. However, smaller volume conventional flow cells also reduce the path length upon which the signal strength depends, according to Beer's law; and a reduction in cross-section means the light path is reduced, and transmission drops increasing noise. Therefore, if a conventional HPLC flow cell is used, UPLC sensitivity would be compromised. So to avoid increasing

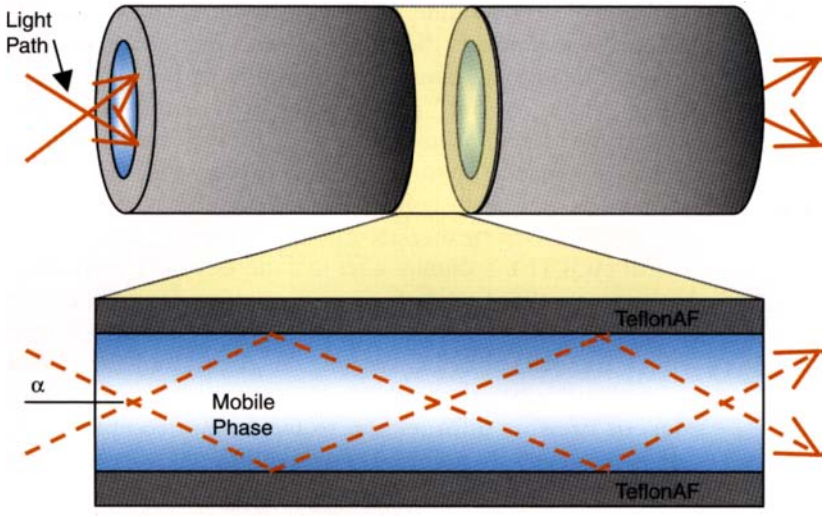

FIGURE 8 **Schematic of the UPLC light-guiding detector flow cell.**

system bandspread and to maintain concentration and sensitivity, a small volume light-guiding flow cell (essentially an optical fiber whose core material is a fluid) was developed, as illustrated in Figure 8. Designed for optimum path length and high light throughput, the cell uses Teflon® AF, an internally reflective surface, to improve light transmission efficiency by eliminating internal absorption. Light is efficiently transferred down the flow cell in an internal reflectance mode that in the analytical flow cell still maintains a 10 mm flow cell path length with a volume of only 500 nL. For high sensitivity work, a second cell is available with a longer path length (25 mm) without introducing additional noise. The light-guiding flow cell design results in exceptional signal to noise ratios, enabling the use of the UPLC system to, for example, determine trace levels of impurities as demonstrated in Figure 9. In this example, the PDA detector allows quantitation of impurities down to 0.0004%.

Combining low noise, wide linear range and excellent spectral resolution at both high and low signal levels is also required for high sensitivity trace analyses. In the typical impurity profile shown in Figure 10, the active ingredient (prilocaine) is present at 1.6 absorbance units (AU), while a 0.01% known impurity (*o*-toluidine) shows an absorbance of 0.002 AU. Both can be quantitated in a single analysis with excellent spectral resolution for both, saving time and making the method more robust.

(ii) Data Rate and Filtering Constants. With 1.7 μm particles, half-height peak widths of less than one second are obtained, posing significant challenges for the detector. In order to accurately and reproducibly integrate an analyte peak, the detector sampling rate must be high enough to capture enough data points across the peak, generally 25–50 for

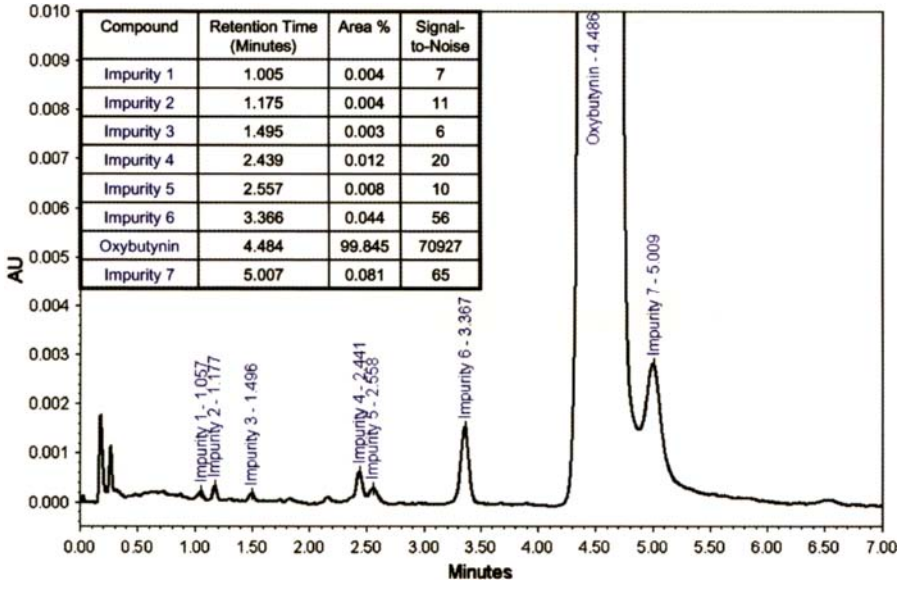

FIGURE 9 **Detector cell sensitivity illustrated by a trace level impurity determination by UPLC. Separation was performed on a 2.1 by 50 mm 1.7 µm ACQUITY BEH C18 column operated at 45°C. An isocratic mobile phase of 65/35 10 mM phosphate buffer, pH 7.0/acetonitrile at a flow rate of 0.800 mL/min was used, with UV detection at 225 nm and 10 pts/s. Sample was 2 mg/mL oxybutynin dissolved in 50/50 water/acetonitrile; 2.1 µL injection.**

reproducible quantitation, as highlighted in Figure 11. Typically, detectors have a base sampling rate which is generally the highest data rate available. In order to get the lower data rates, points are averaged to give the lower data rate. For example, if the base data rate is 20 Hz, to get 10 Hz every 2 data points are averaged, to get 5 Hz, every 4 data points are averaged. The resulting "filtered" data gives lower noise levels and lower data rates. However, filtering must be balanced against the need to collect enough data points to adequately quantitate the peak of interest and the desired signal to noise or sensitivity, requiring independent optimization.

In general, the best filtering constant is the reciprocal of the sampling rate. If increased sensitivity is desired, or if the baseline noise is interfering with integration, the filtering constant should be increased. If resolution is compromised, it should be decreased.

(iii) PDA Method Development Example. The PDA detector can be a valuable tool during method development. One of the ways PDA detection proves its usefulness is in tracking peaks during method development, or in the case of Figure 12, tracking peaks when migrating methods from HPLC to UPLC. Sometimes transferring methods from HPLC to UPLC will result in selectivity changes.

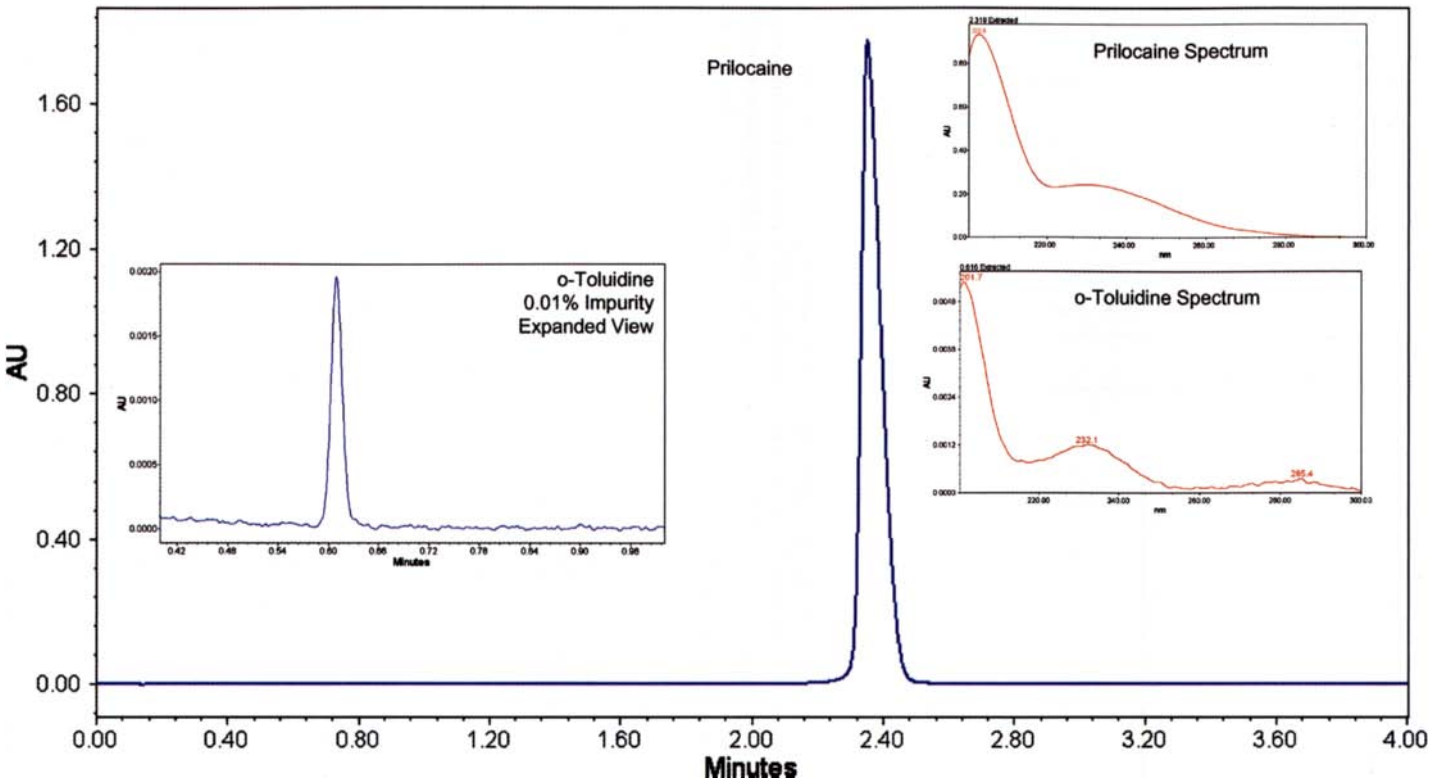

FIGURE 10 High sensitivity PDA detection impurity profile. Separation was performed on a 2.1 by 50 mm 1.7 μm ACQUITY BEH C18 column operated at 40°C. An isocratic mobile phase of 75/25 10 mM ammonium bicarbonate, pH 9.5/acetonitrile at a flow rate of 0.800 mL/min was used, with UV detection at 230 nm and 20 pts/s; 2.1 μL injection. Insets show an expanded section of the baseline to see the impurity, and the corresponding UC spectra extracted from each of the peak apexes.

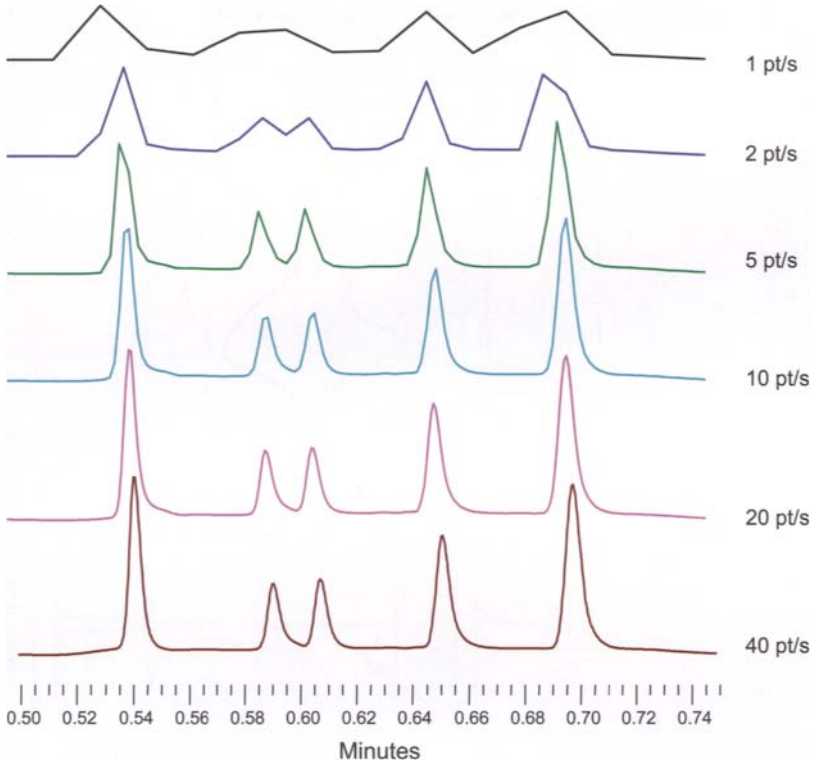

FIGURE 11 **Impact of data acquisition rates. Faster data acquisition rates keep up with the speed of the chromatography for quantifiable data. Faster rates allow the detector to capture enough data points across a very narrow (potentially one second) UPLC peak to meet analytical requirements.**

When properly configured, the spectral matching algorithms can be used to follow peaks as selectivity changes, saving time by negating the need for individual injections.

(b) Mass Spectrometric Detection:

Mass spectrometry is a powerful analytical technique that can be used to confirm, quantify, identify, or characterize compounds of interest. Mass spectrometers measure the mass-to-charge (*m/z*) ratio of ions in the gas phase, allowing the determination of a compound's molecular weight (to varying degrees of accuracy). By breaking apart molecules into fragments, MS can also be used to analyze smaller portions of a molecule. Information from this fragmentation assists in the elucidation of the compound's chemical structure and properties.

Modern mass spectrometers are simple, easy to use instruments with a much smaller footprint than their predecessors and can be configured with a chromatographic method development system to provide a wealth

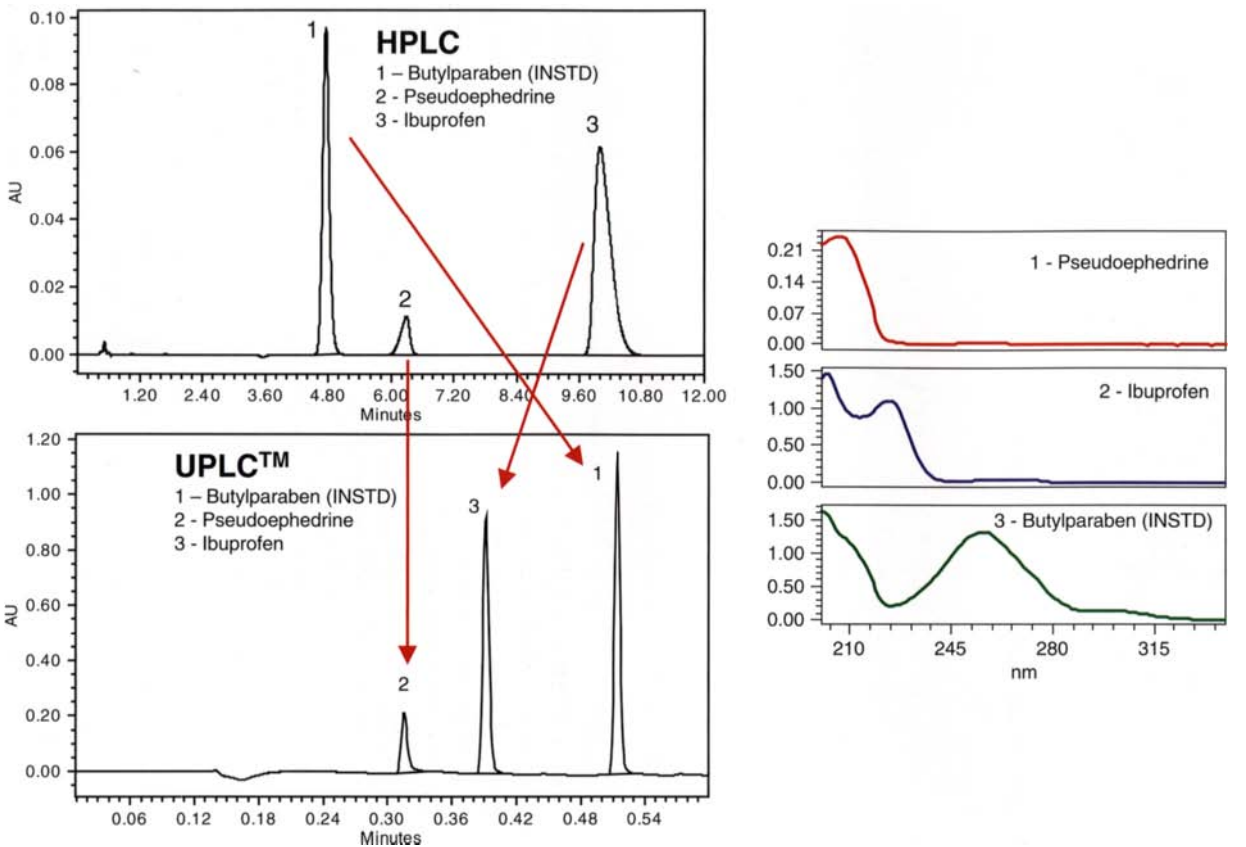

FIGURE 12 Using the PDA detector to track peaks during method development. Although selectivity changes from run to run (top to bottom), the spectra shown on the right can be stored in a library and used to identify the individual peaks, rather than for example injecting individual standards.

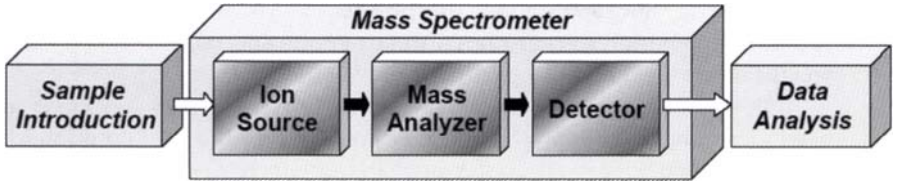

FIGURE 13 The basic components of an MS system.

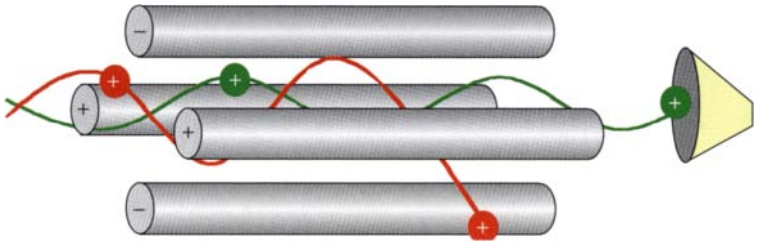

FIGURE 14 Quadrupole MS schematic.

of useful information. The basic components of an MS system are shown in Figure 13. When it comes to MS detection, the low system and dwell volume of the UPLC system increases peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting) and promotes increased source ionization efficiencies. Combined with the added resolution, this makes UPLC the ideal inlet technology for MS in the method development laboratory.

A comprehensive treatment of MS is outside the scope of this chapter. Many excellent detailed reviews of the technology are available.[17,18] But single quadrupole mass spectrometers are becoming increasingly common in the method development laboratory and will be covered here in some detail.

Quadrupole MS uses radio frequency (rf) and direct current (dc) voltages for the separation of ions, and they are probably the most widespread mass spectrometers because of their relatively low price and ease of operation. In a quadrupole mass spectrometer the rf and dc potentials are applied to four rods arranged in a square array, as illustrated in Figure 14. Ions are scanned or filtered by varying the dc/rf voltages across the quadrupole rods. Generally speaking, quadrupole analyzers are used to determine the nominal mass of a compound. Nominal mass is often used to confirm the identity of known compounds in method development.

In method development, MS is used in much the same way as the PDA, to identify and track peaks as selectivity changes, and to monitor

for co-elution. But unlike PDA, MS provides a positive identity, and can provide deconvoluted total ion chromatograms specific for a molecular weight when co-elution of partial resolution does occur.

Of course, no detector response is universal. MS response is dependent on the ability to ionize a compound, and not all compounds can be ionized under all conditions. In similar respects, not all compounds have UV chromophores, so PDA detection is of course limited. However, it is very rare to have both no ionization and lack of a UV chromophore; therefore it is increasingly common to use both MS and PDA in tandem during method development.

When it comes to MS detection, the low UPLC system and dwell volume increases peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting) and the added resolution promotes increased source ionization efficiencies, making UPLC the ideal technology for an MS inlet. Higher UPLC sensitivity also improves the quality of the spectra obtained.

(i) Mobile Phase and Column Compatibility Considerations. Using a mass detector places constraints on mobile phase selection. These constraints are different from what is to be considered with other detectors. Proper selection of the mobile phase and any additives are critical to detection viability.

First and foremost the mobile phase must be suitable for the ionization, and must be selected depending on the ionization mode (electrospray (ESI) or atmospheric pressure chemical ionization (APCI), positive or negative mode) and the analyte (e.g. pK_a). The molecular weight of the mobile phase components should also be considered. It is not possible to analyze compounds whose molecular weight is lower than that of the mobile phase solvents or any additives. For routine operation, it is easier to use volatile buffers. Acids like HCl, H_2SO_4, or methane sulfonic acid might damage the instrument and should not be used. Volatile organic acids (e.g. TFA, formic, acetic) should be used instead.

Some ions (e.g. Na, NH_4, acetate) from the mobile phase can form adducts. In the case of phosphate, multiple adducts are observed, which can produce complicated mass spectra. The formation of adduct is usually not a reason for avoiding a mobile phase as adducts can sometime be used to advantage. Ion pairing reagents can impact the spray formation, the droplet evaporation, and compete in terms of ion formation, and are generally avoided. Buffer concentration is generally kept as low as possible (mM range). If the buffer concentration is too high, ion suppression occurs effecting sensitivity. Common eluents for LC/MS include methanol/water, acetonitrile/water (methanol usually gives a better sensitivity than acetonitrile), pH modifiers: formic, acetic acids, TFA, NH_4, TEA, DEA, and buffers: carbonates, ammonium formate, ammonium acetate, ammonium carbonates, and ammonium phosphate (all non-volatile). The column, while of course providing the separation,

without using a high concentration of buffers, or ion pairing reagents, must be stable so that the column will not "bleed" or shed interfering compounds. Special low or no-bleed MS versions of columns are available from most suppliers.

IV. MIGRATING METHODS FROM HPLC TO UPLC

A. Method Development

When it comes to developing methods for UPLC, the approach is really no different from that traditionally used for HPLC, since UPLC is based upon the same underlying chromatographic theory. But given the speed, and often improved resolution of UPLC, method development is accomplished a whole lot faster.

During method development selectivity can be manipulated by any one or a combination of different factors that include solvent composition, type of column stationary phase, and mobile phase buffers and pH. Chromatographers for the most part are comfortable changing solvents and column stationary phases to generate a separation. However, advances in LC column technology recently have made possible the use of pH as a true selectivity tool for the separation of ionizable compounds. Hybrid-chemistry UPLC columns take advantage of the best of both the silica and polymeric column worlds. They are manufactured using a classical sol–gel synthesis that incorporates carbon in the form of methyl groups, resulting in columns that are mechanically strong, with high efficiency, and operate over an extended pH range. The graphics in Figure 15 illustrate why pH can be such a useful tool. Acidic compounds are more retained at low pH, while basic compounds are more retained at higher pH (neutral compounds are of course unaffected). At pH values used traditionally (pH 4–8), a slight change in pH would result in a dramatic shift in retention (up-slope or down-slope of curve). However, by operating at pH extremes, not only is there a 10–30-fold difference in retention that can be exploited in method development, the method can be made more robust as well, a desirable outcome with validation in mind. Indeed, the selectivity differences afforded by a change in pH are the equivalent to a 20% change in the organic solvent composition, and often are underutilized.

Faster, higher resolution UPLC separations can cut method development time from days, to hours, or even minutes. Figure 16 is an example of an UPLC separation of several closely related coumarins that was developed in under an hour; including UPLC scouting runs for gradient optimization, and individual runs for elution order identification and PDA spectral library creation. These runs were performed in a fraction of the time that would be necessary by conventional HPLC, resulting in significant time savings. To develop the method illustrated in Figure 16,

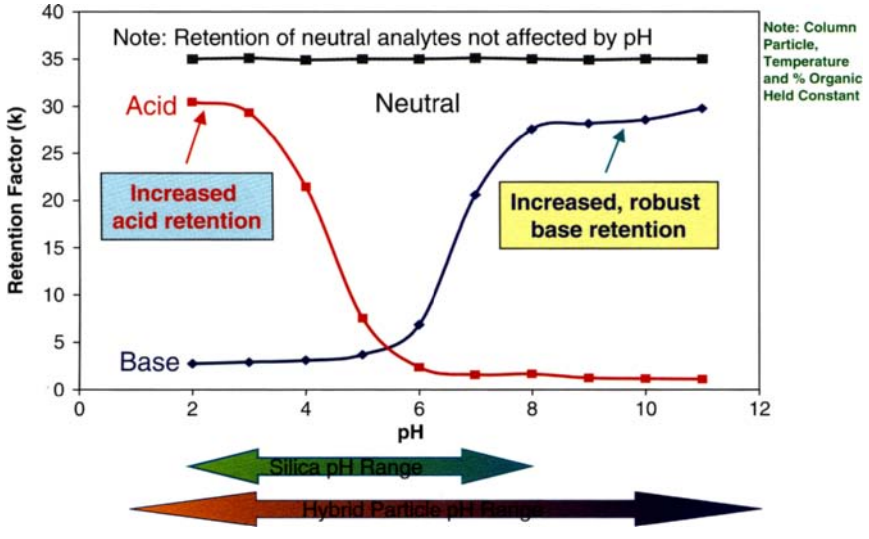

FIGURE 15 Reversed phase retention behavior as pH is varied.

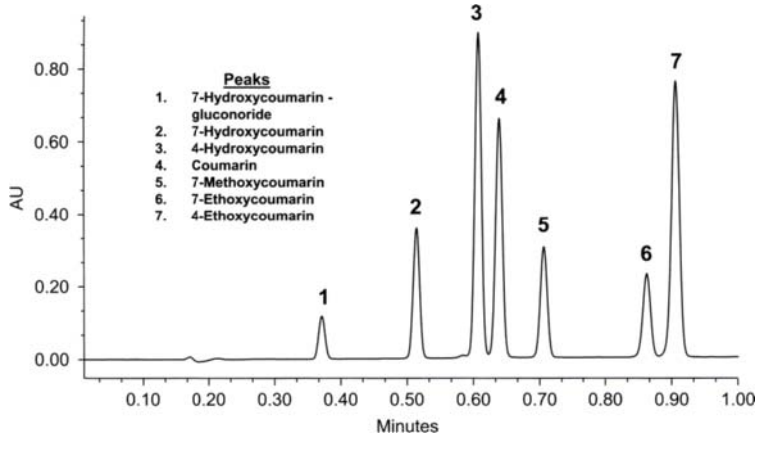

FIGURE 16 UPLC separation of coumarin and related compounds illustrating fast method development. Column: 2.1 by 50 mm 1.7 μm ACQUITY UPLC BEH C18 at 35°C. A 5–80%B linear gradient over 1.0 min, at a flow rate of 1.0 mL/min was used. Mobile phase A was 0.1% formic acid, B was acetonitrile. UV detection at 254 nm and 40 pts/s.

two initial generic scouting gradients were run, and then gradient slope and starting organic percentage were adjusted in several subsequent 1.5 min runs, essentially optimizing the method "on the fly". Individual injections of each of the seven compounds were made for peak elution order identification, and a final confirmation run was made, all in under

60 min. The speed of UPLC analyses allows analysts to investigate more conditions during method development, potentially resulting in more robust methods.

B. Method Transfer from HPLC to UPLC

Methods can certainly be developed from the beginning for UPLC. However, if an HPLC method already exists, to take advantage of the throughput, improved resolution, and sensitvity of UPLC, it will be necessary to migrate methods, or transfer them from one technique to the other, which can at first appear challenging. However, by selecting the proper column chemistry and dimensions, and properly scaling injection volume, flow rate, and gradient conditions (if appropriate), the task of migrating methods is really quite easy and straightforward.

1. Selecting the Right Column

A 1.7 μm particle packed column provides significant improvements in resolution because efficiency is better. Separation of the components of a sample, however, still requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations: ACQUITY UPLC™ BEH C18 and C8 (straight chain alkyl columns), ACQUITY UPLC BEH Shield RP18 (embedded polar group column), and ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl) as shown in Figure 17. Each column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability, and chemical interaction with analytes.

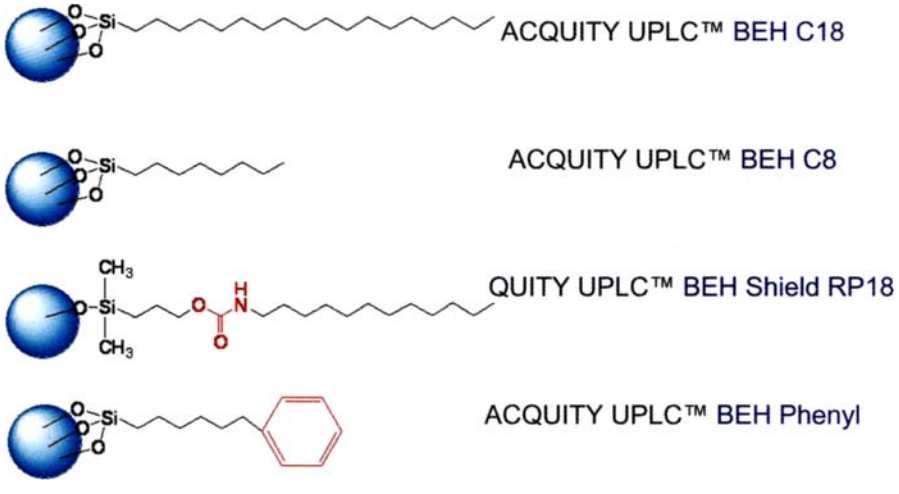

FIGURE 17 ACQUITY BEH column chemistries.

ACQUITY UPLC™ BEH C18 and C8 columns are the universal columns of choice for most UPLC™ separations. They incorporate trifunctional ligand bonding chemistries which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7 μm BEH particle to deliver a wide usable pH operating range. ACQUITY UPLC™ BEH Shield RP18 columns are designed to provide selectivities that complement the C18 and C8 phases. The sheild technology incorporates an embedded carbamate group into the bonded phase ligand, resulting in alternate selectivity and excellent peak shape. ACQUITY UPLC™ BEH Phenyl columns utilize a trifunctional C6 alkyl tether between the phenyl ring and the silyl functionality. This ligand, combined with the same proprietary endcapping processes as the C18 and C8 columns, provides long column lifetimes and excellent peak shape. This unique combination of ligand and endcapping on the 1.7 μm BEH particle creates a new dimension in selectivity allowing a quick match to the exisitng HPLC column. Column selectivity charts plotting selectivity vs. retentivity are commonly used to match existing column chemistries to those used previously.

Having chosen a chemistry, column dimensions are chosen based upon the goals of the analysis. Generally an internal dimension (ID) of 2.1 mm is used in UPLC, unless limited sample is available, or there is a desire to flow directly into a mass spectrometer; in which case a 1.0 mm ID is recommended. In terms of length; for maximum resolution, choose a 100 mm length; for faster analyses, and higher sample throughput, choose 50 mm.

2. Scaling the Separation

Having selected or matched the column chemistry it is now necessary to take a few easy steps using a few equations that geometrically scale the original method to the new column using exactly the same mobile phase composition. These equations take into account the changes in the gradient time (unless using isocratic conditions), flow rate, and injection volume.

The gradient is scaled from HPLC to UPLC using

$$L_2/L_1 \times t_{g1} = t_{g2}$$

where L_1 and L_2 are the lengths of the HPLC and UPLC columns, and t_{g1} and t_{g2} the times of each gradient step respectively.

Flow rate is scaled taking into account the difference in the diameter of the two columns:

$$(d_2)^2/(d_1)^2 \times F_1 = F_2$$

where d_2 and d_1 are the column diameters and F_1 and F_2 the flow rates.

To keep the column volumes proportional, the gradient steps should be re-adjusted for the new flow rate:

$$(F_2 \times t_{g2})/F_3 = t_{g3}$$

where F_2 and t_{g2} are the flow rate and gradient time of the geometrically scaled values (typically 650 µL/min for small molecules on a 2.1 mm ID column) and F_3 and t_{g3} the optimized values.

The injection volume is scaled taking into account the volumes of the two columns:

$$V_1 \times [(r_2^2 \times L_2)/(r_1^2 \times L_1)] = V_2$$

where r_2^2 and r_1^2 are the radii of the columns, L_1 and L_2 the lengths of the columns, and V_1 and V_2 the injection volumes.

As an example, Figure 18 shows an HPLC separation of a series of related caffeic acid derivatives from *Echinacea purpurea*, a natural product. When column re-equilibration is taken into account, the run time exceeds 40 min. When properly scaled for injection volume, flow rate, and gradient time, the UPLC separation illustrated in Figure 19 is obtained. By UPLC, the run time is complete in under 6 min including re-equilibration, increasing throughput approximately 7×, while using

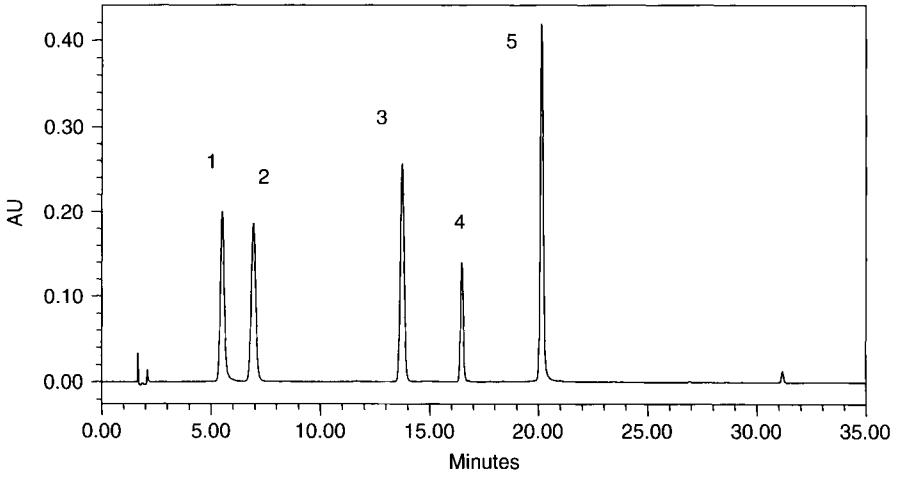

FIGURE 18 **Original HPLC separation of caffeic acid derivatives from *Echinacea purpurea*, a natural product. Column: 4.6 by 150 mm 5.0 µm XTERRA MS C18 at 40ºC. A 8–50%B linear gradient over 32 min, followed by a 3 min step to 90%B, and a 6 min re-equilibration to starting conditions, at a flow rate of 1.0 mL/min was used. Mobile phase A was 0.1% CF3COOH in H2O. Mobile Phase B: 0.08% CF3COOH in acetonitrile, UV detection at 330 nm. Peaks are in order: caftaric acid, chlorogenic acid, cynarin, echinacoside, cichoric acid, 0.1 mg/mL each in 50:50 H2O:MeOH with 0.05% CF3COOH, 10 kL injection.**

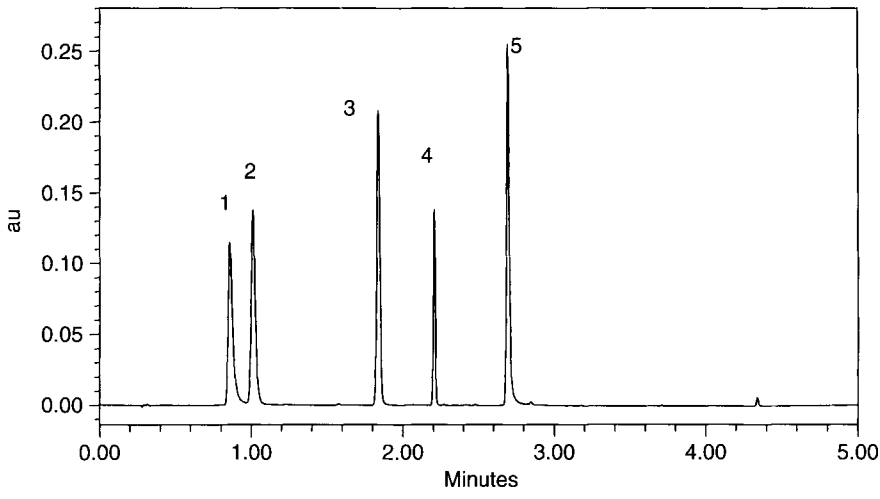

FIGURE 19 **Resulting UPLC separation of caffeic acid derivatives from *Echinacea purpurea*, a natural product, after scaling the HPLC separation. Column: 2.1 by 50 mm 1.7 km ACQUITY BEH C18 at 408C. A 8–50%B linear gradient over 4.45 min, followed by a 0.41 min step to 90%B, and a 1.14 min re-equilibration to starting conditions, at a flow rate of 0.5 mL/min was used. Mobile phase A was 0.1% CF3COOH in H2O. Mobile Phase B: 0.08% CF3COOH in acetonitrile, UV detection at 330 nm. Peaks are in order: caftaric acid, chlorogenic acid, cynarin, echinacoside, cichoric acid, 0.1 mg/mL each in 50:50 H2O:MeOH with 0.05% CF3COOH, 1.0 μL injection.**

about 10× less solvent. All this without really changing the look of the separation; if it was not for the time scale in the figures, it would be difficult to distinguish between the HPLC and UPLC separations.

However, it should also be noted that the increased speed of UPLC does not compromise resolution or sensitivity, as highlighted in Figure 20. By taking advantage of the smaller particle size of the ACQUITY BEH column chemistry, and the low dispersion of the ACQUITY UPLC system, it is possible to dramatically reduce analysis times, while maintaining or even increasing resolution and sensitivity.

V. UPLC APPLICATIONS IN PHARMACEUTICAL ANALYSIS

A. Speed and Peak Capacity

Chromatographers are used to making compromises; and one of the most common scenarios involves sacrificing resolution for speed. With UPLC, resolution and speed compromises are no longer necessary as depicted in Figure 21; a separation of eight diuretics in under 1.6 min. The same separation on a 2.1 by 100 mm 5 μm C18 HPLC column yields almost identical resolution, but takes 10 min. For some analyses,

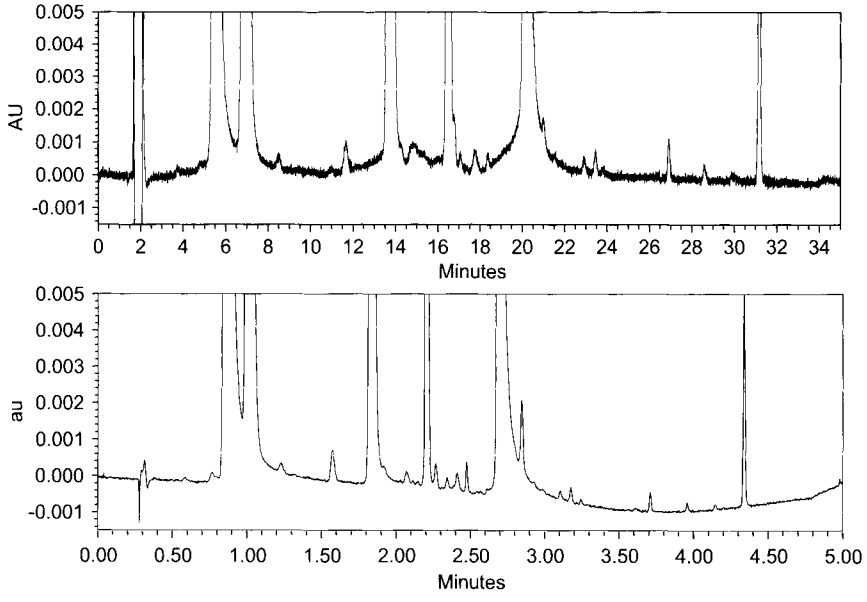

FIGURE 20 **Baseline enhanced view of Figures 17 (HPLC-top) and 18 (UPLC-bottom) showing increased UPLC resolution and signal to noise (sensitivity).**

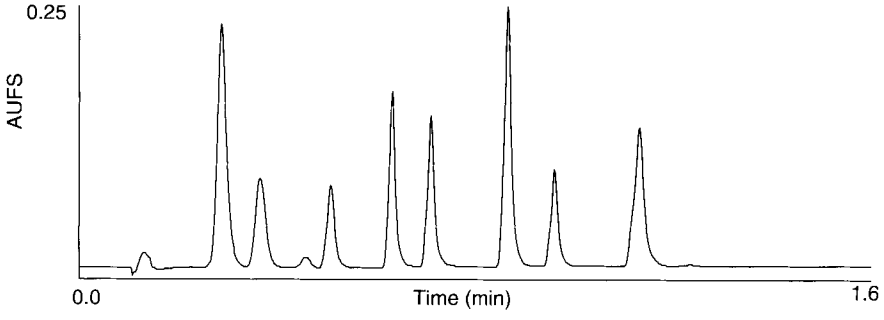

FIGURE 21 **UPLC Separation of eight diuretics. Column: 2.1 by 30 mm 1.7 μm ACQUITY UPLC BEH C18 at 35°C. A 9–45%B linear gradient over 0.8 min, at a flow rate of 0.86 mL/min was used. Mobile phase A was 0.1% formic acid, B was acetonitrile. UV detection at 273 nm. Peaks are in order: acetazolamide, hydrochlorothiazide, impurity, hydroflumethiazide, clopamide, trichlormethiazide, indapamide, bendroflumethiazide, and spironolactone, 0.1 mg/mL each in water.**

however, speed is of a secondary importance; peak capacity and resolution take center stage. Figure 22 shows a peptide map where the desired goal is to maximize the number of peaks. In this application, the increased peak capacity (number of peaks resolved per unit time) of UPLC dramatically improves the quality of the data resulting in a more definitive map.

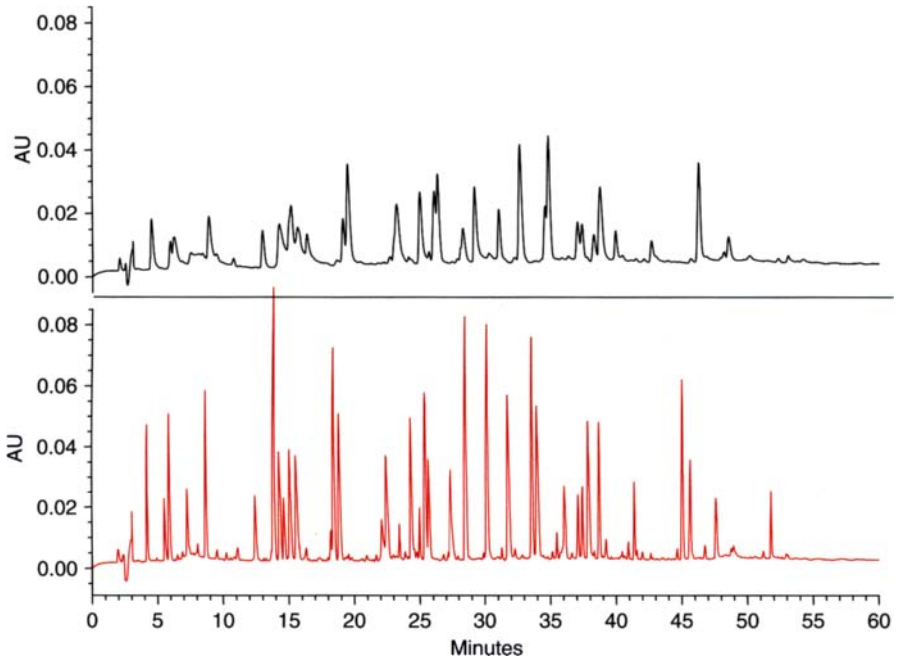

FIGURE 22 HPLC vs. UPLC peak capacity. In this gradient peptide map separation, the HPLC (top) separation (on a 5 μm C18 column) yields 70 peaks, or a peak capacity of 143, while the UPLC separation (bottom) run under identical conditions yields 168 peaks, or a peak capacity of 360, a 2.5× increase.

B. Peptide Mapping

Peptide mapping continues to be the preferred technique for the comprehensive characterization of biopharmaceutical products. Its applications include: the identification of proteins based on the elution pattern of the peptide fragments, the determination of post-translational modifications, the confirmation of genetic stability, and the analysis of protein sequence when interfaced to a mass spectrometer. In a peptide map, it is necessary to resolve each peptide fragment into a single peak. Therefore, peptide mapping represents a significant chromatographic challenge, due to the inherent complexity of peptide digests. In addition to the large number of peptides that are generated from the enzymatic digest of a protein, the number of alternative peptide structures (post-translational modifications, oxidations, etc.) can be significant. The capabilities of UPLC technologies make higher resolution peptide mapping possible.

The chromatographic benefits of UPLC are largely derived from reduced band broadening that is, in turn, a consequence of reduced diffusion distances in small particles. This process is quantitatively

described in the well known van Deemter equation that relates height equivalent of a theoretical plate to linear velocity. In practical terms, these principles suggest that the smaller particles used in UPLC could double the resolving power in a peptide mapping experiment and could simultaneously reduce the separation time because the optimum is achieved at a higher linear velocity. Smaller particles can therefore improve both resolution and sensitivity by reducing diffusion-related band broadening, while at the same time reducing analysis times, without compromise.

The following examples serve to illustrate these points.

1. Improved Resolution and Sensitivity

Peptide maps run by HPLC often require cycle times as long as 3–5 h to separate all the peptides within the digest, especially for large proteins like antibodies. While faster peptide maps would be desirable, it is critically important that resolution not be compromised to ensure that the test results provide the same level of information.

In Figure 23, the separation of a tryptic digest of enolase is shown with a 3.5 μm C18 HPLC column with 300 Å pores, typical of the most common peptide separation columns, and a 1.7 μm UPLC column under identical conditions. In the UPLC separation, more peaks are observed, and the overall resolution and sensitivity are higher. In the UPLC map, there are several small peaks that are difficult to discern in the HPLC map. This result demonstrates that UPLC offers higher resolution and sensitivity when compared to HPLC under the same gradient conditions.

The higher resolution and sensitivity with UPLC is particularly important when the peptide map is used to detect modified peptides. Higher resolution ensures that modified peptides are resolved from the unmodified form, as well as from other peptides in the digest. Higher sensitivity means that modified peptides can be detected at lower levels; where, for example, in Figure 24, UPLC is used to separate a deamidated peptide from its unmodified form.

2. Increasing Speed

To demonstrate how UPLC can resolve the same number of peaks in a peptide map as HPLC but in less time, the separation of an enolase digest was done on a 50 mm length UPLC column with a 20 min gradient and on a 100 mm length HPLC column with a 40 min gradient, both with flow rates of 100 μL/min. These chromatograms are shown in Figure 25. The increased efficiency of the smaller UPLC particles means shorter columns can be used, shortening run times. The UPLC separation shows the same number of peaks and a similar overall elution pattern as the HPLC separation, but in half the time, demonstrating that UPLC offers the potential to reduce cycle times for peptide maps.

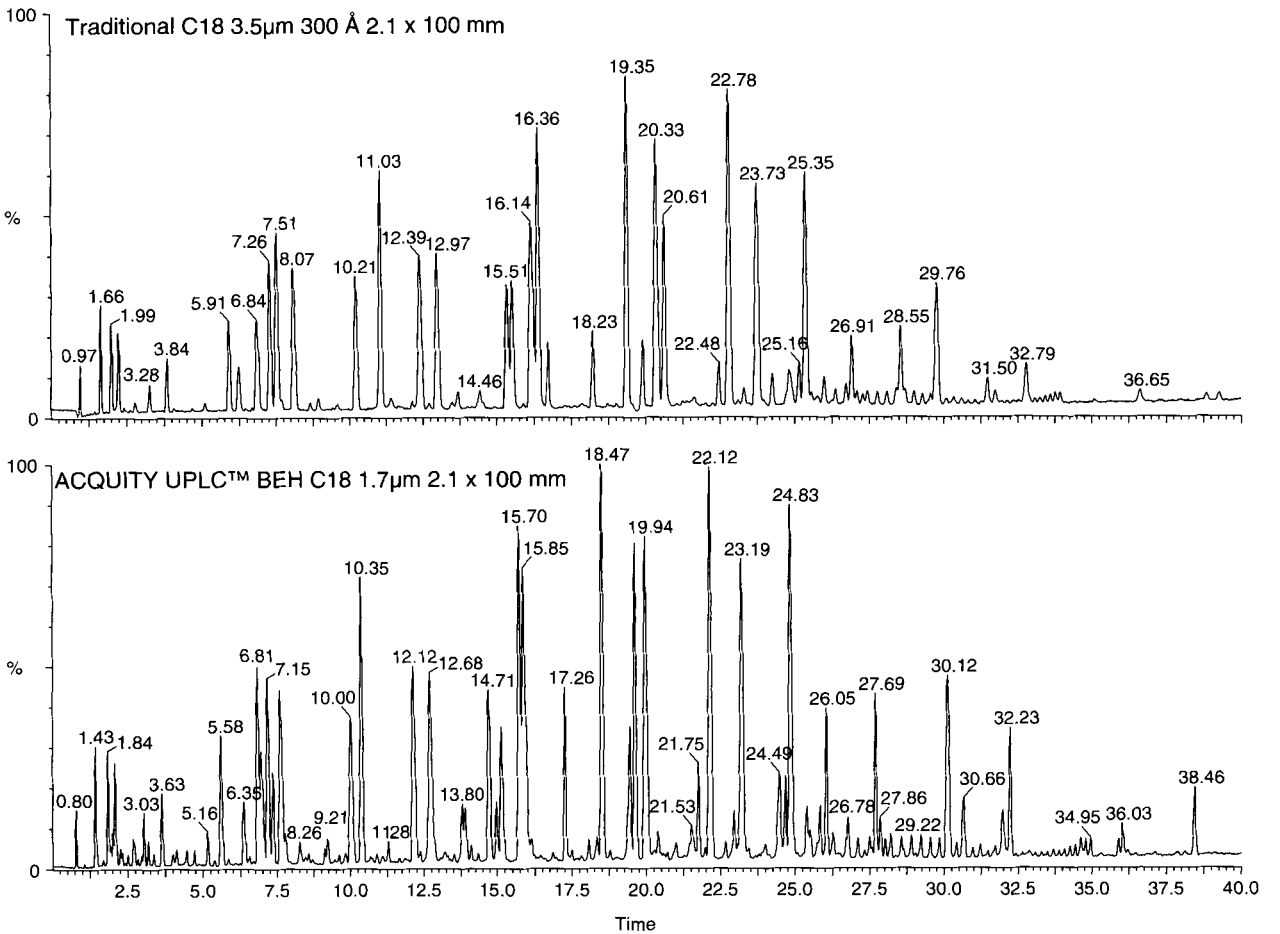

FIGURE 23 **Suitability of UPLC for peptide mapping.**

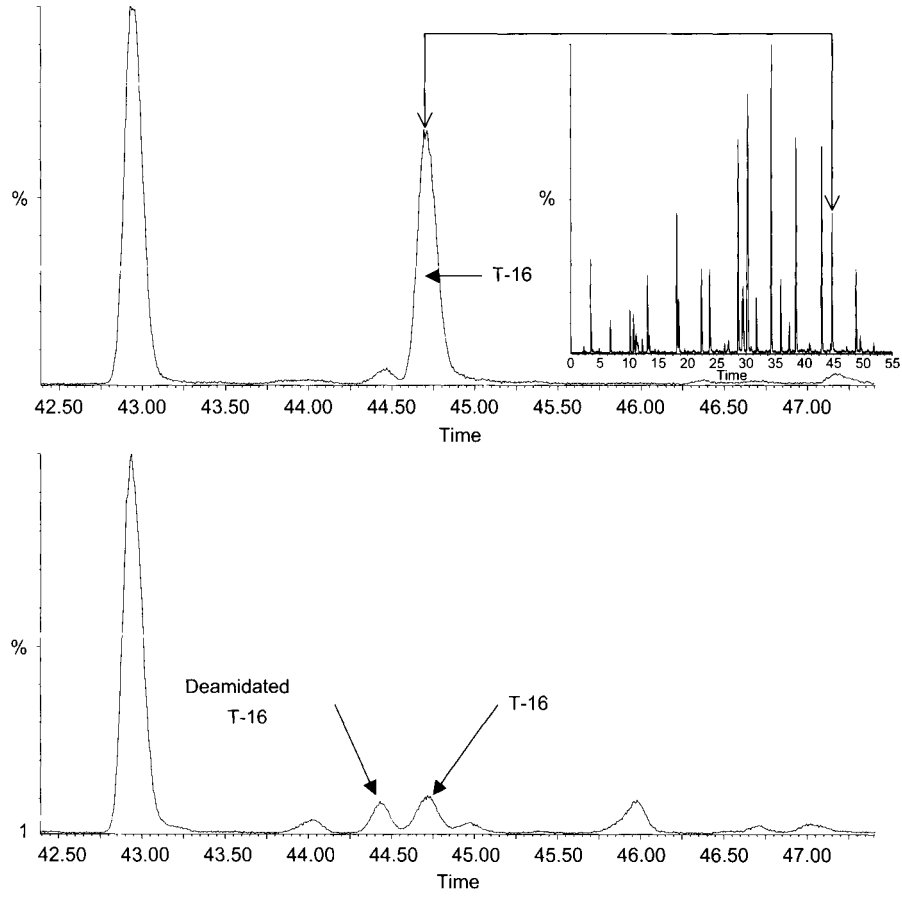

FIGURE 24 **Separation of a deamidated peptide from its native form.**

3. Post-Translational Modifications

Glycosylation is an important post-translational modification that plays a critical role in determining the efficacy and safety of a therapeutic protein. Glycosylation can be analyzed on the intact protein by MS, as released glycans by a variety of techniques, or as glycopeptides in LC/MS peptide maps. When glycosylation can be characterized with LC/MS of the glycopeptides, the site of attachment can be directly determined and structural information can be obtained through MS/MS experiments. This approach is often limited, however, by the poor chromatographic peak shape of glycopeptides and incomplete resolution of glycoforms with HPLC peptide mapping. The poor peak shape has been attributed to the large size of the glycopeptides and their heterogeneous structure. Figure 26 shows the UPLC/MS separation of a tryptic digest of β-1 acid

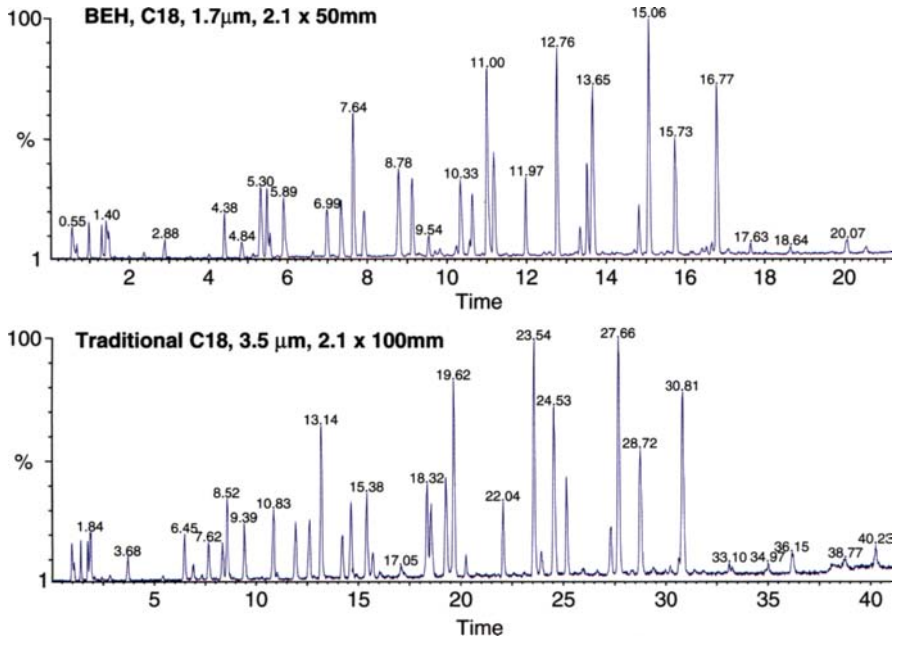

FIGURE 25 **Reducing column length for increased speed.**

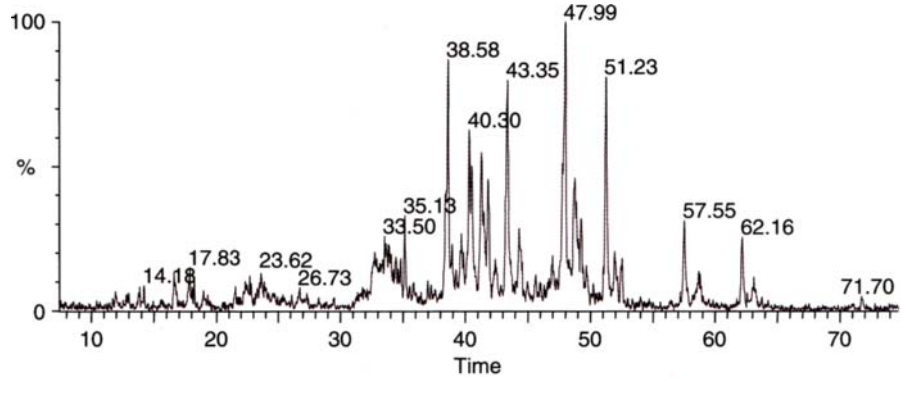

FIGURE 26 **A glycopeptide UPLC separation.**

glycoprotein. The MS detection was performed with a Q-TOF mass spectrometer, which is well-suited for glycopeptides due to its extended mass range. Data is plotted as a selected ion chromatogram for *m/z* 657, a signature ion for glycopeptides resulting from carbohydrate fragments. The glycopeptides are detected as sharp, symmetrical peaks with UPLC. These characteristics are important for minimizing spectral overlap of different

glycoforms of the same peptide. UPLC combined with ESI/TOF MS will be a powerful tool for studying the glycosylation state of proteins.

UPLC facilitates notable improvements in peptide mapping when compared to HPLC. Better resolution is obtained in combination with generally increased sensitivity. Run time can be reduced without compromising resolution by reducing column length and increasing the flow rate. Selectivity is comparable to that of common reversed phase HPLC peptide mapping columns and methods can be easily transferred from HPLC to UPLC and easily interfaced to ESI-MS. UPLC with ESI/TOF is especially suitable for separation of glycopeptides and clearly proving to be the next generation tool for peptide mapping.

C. MS Detection

The success of the drug discovery process is heavily dependent upon the early prediction of metabolic fate and interactions of drug candidate molecules. To prevent "poor" compounds from progressing through the discovery process, factors such as metabolic stability, toxic metabolite production, p450 inhibition, and induction are all routinely monitored. By the mid-1990s, HPLC directly coupled to MS was in routine use in drug metabolism laboratories for these types of studies.[19-25] Enhanced selectivity and sensitivity, and rapid, generic gradients made LC/MS the predominate technology for both quantitative and qualitative analyses. However, with the ever increasing numbers and diversity of compounds entering development, and the complex nature of the biological matrices being analyzed, new analytical procedures and technology were required to keep pace with the testing demands. Unexpected, reactive, or toxic metabolites must be identified as early as possible to reduce the very costly attrition rate. This quest for more accurate data meant improving the chromatographic resolution to obtain higher peak capacity, reducing the co-elution of metabolites, while enhancing the sensitivity and decreasing ion suppression in the MS.

When it comes to MS detection, the low system and dwell volume increases peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting) and promotes increased source ionization efficiencies. Combined with the added resolution, UPLC is an ideal technology for in vitro drug metabolism studies as illustrated by the analysis of the in vitro metabolism of dextromethorphan. Dextromethorphan undergoes O-dealkylation in two positions leading to three major phase I metabolites. These products can be further metabolized via conjugation with glucuronic acid to form metabolites of masses $MH^+ = 434$ and 420. The data in Figure 27a and b shows the HPLC-MS and UPLC-MS separations respectively of the in vitro incubation of dextromethorphan with rat liver microsomes. As shown the chromatographic performance of the 1.7 μm particles is significantly better than that produced by the 3.5 μm material. The 1.7 μm material gives peaks

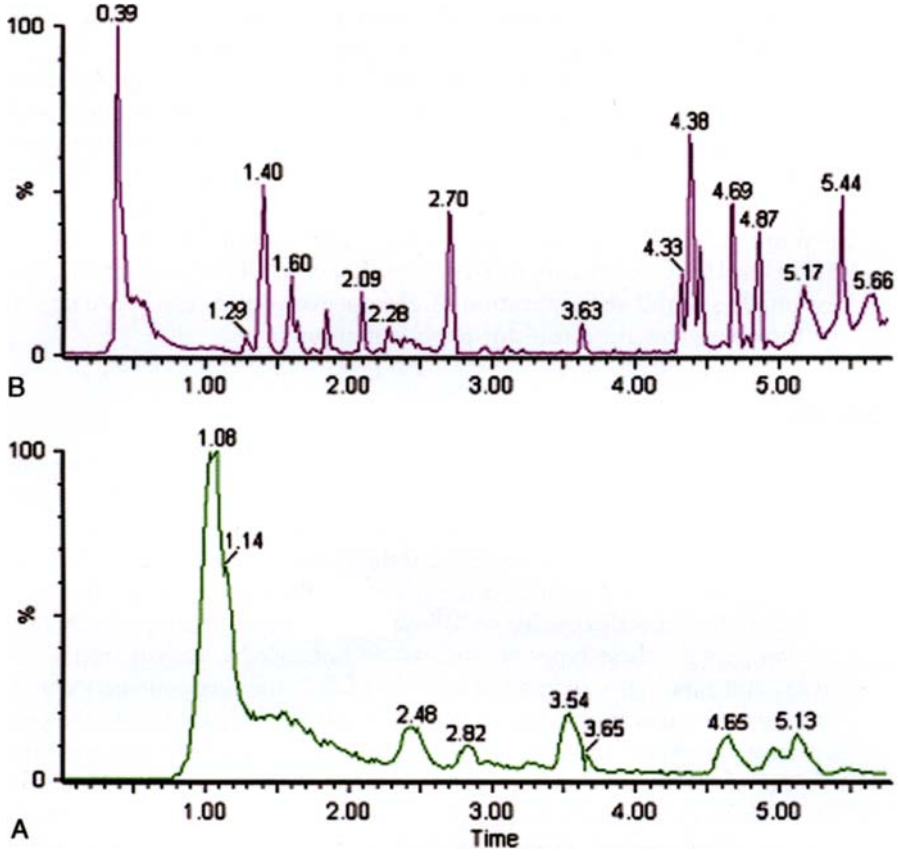

FIGURE 27 **Full scan TOF MS for HPLC (a) and UPLC analyses (b) of the metabolites of dextromethorphan.**

of width 4 s at the base, resulting in a peak capacity of over 100, whereas with HPLC the average peak width was 20 s at the base giving a total peak capacity of just 20, resulting in a fivefold increase in the performance of the UPLC system. The extracted ion chromatogram m/z = 258 and m/z = 244 for the HPLC/MS analysis is shown in Figure 28.

In Figure 28 we can see the two O-dealkylated metabolites of dextromethorphan at m/z = 258, resolved to about 80%, while the 244 metabolite is barely visible. These results can be compared to those obtained by UPLC, here we can see that the two 258 ions are clearly resolved and that the 244 ion is now easily detected, as illustrated in Figure 29.

This data clearly illustrates the improved resolution and sensitivity of the UPLC system. This extra resolution is particularly important when analyzing isobaric compounds such as these dealkylated metabolites. Without the resolution generated by UPLC it would be possible to falsely assign the structure of a metabolite or miss a potential toxic moiety. The

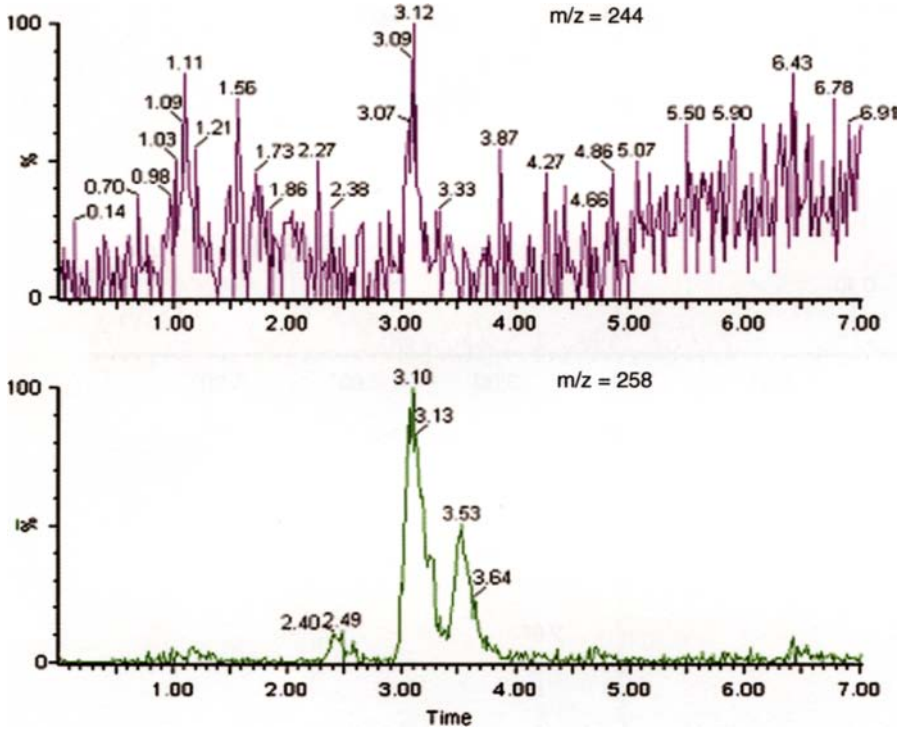

FIGURE 28 **Extracted ion chromatograms for major N and O dealkylated and double dealkylation metabolites of dextromethorphan by HPLC/TOF MS.**

extra sensitivity produced by the UPLC system ensures more low concentration metabolites will be detected, helping to prevent potentially toxic compounds from progressing further into the drug discovery process. This extra sensitivity is extremely important when performing MS/MS experiments as it can make the difference between obtaining an interpretable spectrum or not. The increased resolution and sensitivity has enabled the detection of more drug metabolites than that previously possible, and reduced the possibility of missing other important biotransformations. The increased peak sensitivity will not only allow more metabolites to be detected but it will enhance the quality of any MS/MS spectra acquired. With better results resulting in higher quality data, UPLC greatly aids the researcher in making better decisions faster.

VI. SUMMARY AND CONCLUSIONS

UPLC using 1.7 μm particles, and a properly, holistically designed system provides significantly more resolution while reducing run times, and improving sensitivity for the analyses pharmaceutical compounds.

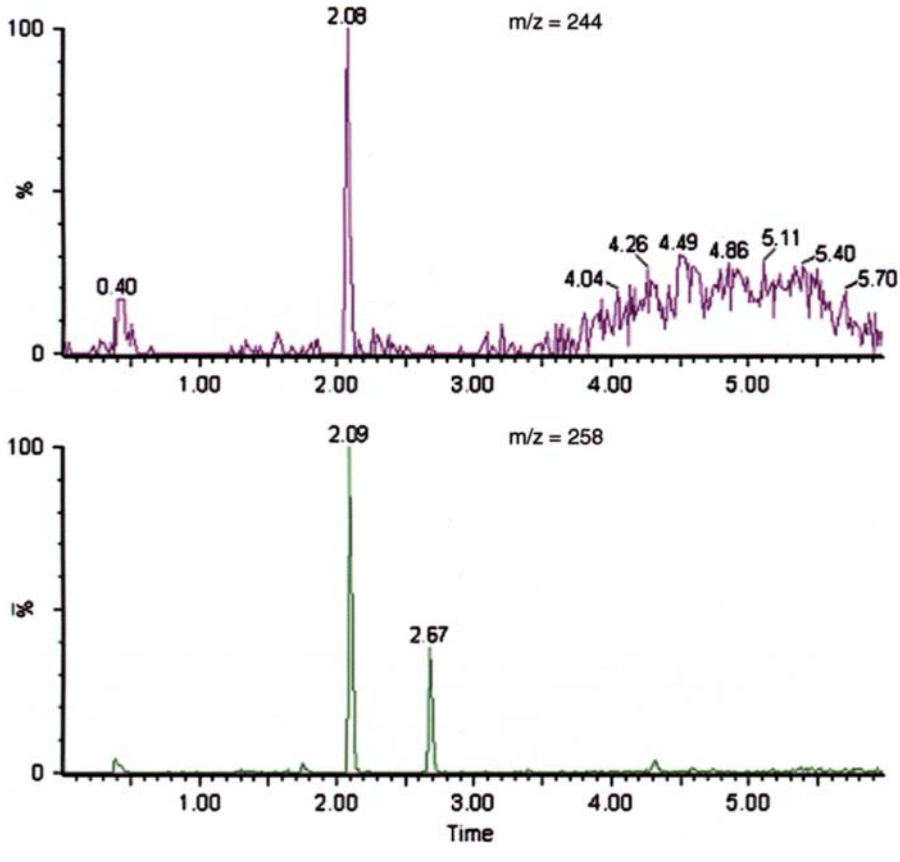

FIGURE 29 **Extracted ion chromatograms for major N and O dealkylated and double dealkylation metabolites of dextromethorphan by UPLC/TOF MS.**

At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography. New technology in terms of column chemistry and instrumentation boosts productivity by providing more information per unit of work as UPLC fulfills the promise of increased resolution, speed, and sensitivity predicted for liquid chromatography.

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7
HYPHENATED TECHNIQUES

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ABSTRACT

This chapter discusses the combination of high performance liquid chromatography (HPLC) with compound specific detection by mass spectrometry (HPLC/MS or LC/MS), and describes the theory and application of modern LC/MS in its most commonly employed configurations. Included are discussions of the most widely applied ionization processes for LC/MS, and the most useful and commonly used mass analyzers. Particular emphasis is given to HPLC method development, and the impact of employing LC/MS on the method development process. Also included, is a discussion of gas-phase ion chemistry and how chromatography parameters can be employed to manipulate gas-phase ion chemistry to enhance LC/MS analysis. Finally, the relatively

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new combination of liquid chromatography with nuclear magnetic resonance spectroscopy (LC/NMR) is described and discussed. This discussion includes a critical examination of the costs and benefits of LC/NMR relative to conventional "tube" NMR with off-line sample isolation and purification. Finally, the double hyphenated technique of LC/MS/NMR is introduced.

I. INTRODUCTION AND BACKGROUND

The requirement for sample volatilization imposed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) has led to a significant research interest in LC/MS.[1,31] This combination was realized during the 1980s, and since that time LC/MS has come to occupy a central role in pharmaceutical analysis. Its areas of application include the identification of drug substance and drug product-related impurities and degradation products, the identification of drug-related metabolites at trace levels in biological matrices, the high sensitivity/high selectivity quantitation of drugs and their metabolites in biological matrices, high-throughput screening of potential drug candidates, peptide and protein analysis in support of drug discovery activities, and the list goes on.

Although mass spectrometry includes a family of detection techniques with significant capability for qualitative and quantitative analytical application to pharmaceutical problem solving, nuclear magnetic resonance spectroscopy (NMR) has long been demonstrated to possess significant advantages for qualitative analysis of organic chemical entities.[49,50] Since NMR in its traditional modes of operation uses liquid samples, it was logical to consider the combination of NMR with HPLC (HPLC/NMR or LC/NMR). LC/NMR instruments became available during the 1990s and have seen increasing application in pharmaceutical analysis, primarily for the identification of drug-related impurities and metabolites, but also in areas such as high-throughput screening of drug candidates and other drug discovery applications. More recently, so-called double hybrid LC/MS/NMR instruments have become available and have been applied to pharmaceutical problem solving.

This chapter discusses the combinations of mass spectrometry and nuclear magnetic resonance spectroscopy with high performance liquid chromatography, and the applications of these hyphenated techniques to pharmaceutical analysis. Included, are citations to a number of recent comprehensive review articles related to various pharmaceutical applications. Particular emphasis is given to chromatography method development, and the impact of employing LC/MS, LC/NMR, and LC/MS/NMR on the method development process. The relative costs and benefits of these techniques are also discussed, including a critical

examination of the costs and benefits of LC/NMR relative to conventional "tube" NMR with off-line sample isolation and purification.

II. COMBINED LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

A. Introduction and Background

The complete history of mass spectrometry up to the year 2002, including an historical perspective on LC/MS and its various applications (including pharmaceutical applications) is presented by Grayson.[1] An organic mass spectrometer is an analytical instrument capable of generating ions from neutral organic molecules, separating these ions according to their mass-to-charge ratios, and detecting the resulting mass separated ions to produce a "mass spectrum". Based on this definition, the organic mass spectrometer can be represented by the schematic shown in Figure 1. All organic mass spectrometers must have sample inlet systems (e.g., HPLC), processes for generating ions (i.e., "ion sources"; see Table 1), mass analyzers (e.g., quadrupole mass filters, magnetic sectors, quadrupole ion traps, time-of-flight (TOF) analyzers, etc.; also see Tables 1 and 3), and ion detectors (e.g., electron multipliers, photomultipliers, etc.).

The fundamental problem of interfacing HPLC with MS is the high vacuum system of the mass spectrometer and the fact that the HPLC mobile phase is a liquid, often containing high levels of water, which is relatively high boiling and, therefore, difficult for high vacuum systems to handle. The choice, therefore, is either to remove the

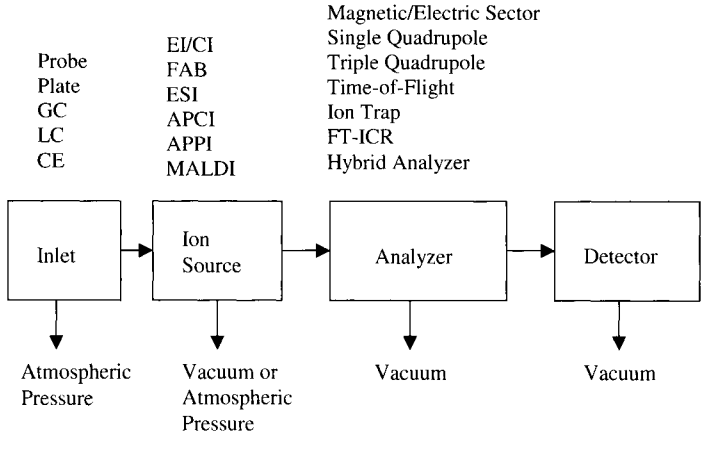


FIGURE 1 Block schematic diagram of a mass spectrometer (CE, capillary electrophoresis; for other acronyms see Table 1).

TABLE I Summary of the Most Significant Ionization Processes and Mass Analyzers Utilized Over the History of Mass Spectrometry (See Reference 1)

Ionization processes	Acronym
Electron ionization (impact)[a]	EI
Chemical ionization[a]	CI
Fast atom bombardment	FAB
Plasma desorption	PD
Laser desorption	LD
Field ionization	FI
Field desorption	FD
Secondary ion	SIMS
Thermospray	TSP
Electrospray[a]	ESI
Atmospheric pressure chemical ionization[a]	APCI
Atmospheric pressure photoionization[a]	APPI
Matrix-assisted laser desorption ionization[a]	MALDI
Mass analyzers	
Magnetic sector/electric sector	BE (or EB)
Quadrupole mass filter (hexapole/octapole)	Q
Triple quadrupole	QQQ
Time-of-flight	TOF
Quadrupole ion trap (cubic or linear)	Trap
Ion cyclotron resonance	ICR (FTMS)
Hybrid sector	EBE; EBEB; BEBE, etc.
Hybrid sector/quadrupole	EBQQ
Hybrid quadrupole/time-of-flight	QTOF
Hybrid time-of-flight	TOF/TOF
Hybrid quadrupole/ion trap	Q-trap
Hybrid FTMS (ICR)	Trap/FTMS; QQFTMS

[a]Ionization processes with broad/current pharmaceutical industry application.

HPLC mobile phase before it enters the vacuum system of the mass spectrometer, or to employ it to assist ionization of analyte molecules and deal with vacuum issues in some other way. Given this, LC/MS interfaces can be grouped into two general categories as shown in Table 2: (a) so-called "transport devices", which were designed to separate the HPLC mobile phase from dissolved analyte molecules prior to ionization; and (b) "unique ionization phenomena" in which the HPLC mobile phase participates in the ionization of dissolved analyte molecules. Transport devices include the "moving belt" and "particle-beam" interfaces, which have seen only limited application in pharmaceutical analysis. Of central historical and current interest

TABLE 2 Current and Historical LC/MS Interfaces (See Reference 1)

Interface designation	Interface type	Comments
Direct liquid introduction (DLI)	Transport device	Designed to interface with an EI/CI ion source; not widely applied
Moving belt (wire)	Transport device	Designed to interface with an EI/CI ion source; not widely applied
Thermospray (TSP)	Ionization process	First LC/MS interface to involve a new ionization phenomenon; First LC/MS interface to be widely applied to pharmaceutical analysis
Particle beam	Transport device	Designed to interface with an EI/CI ion source; some environmental science application
Continuous-flow FAB (CF-FAB)	Transport device	Designed to interface with fast atom bombardment; useful for biomacromolecules; widely applied in drug discovery
Electrospray (ESI)	Ionization process	Facilitated the "universal" pharmaceutical application of LC/MS
Atmospheric pressure chemical ionization (APCI)	Ionization process	Partner to electrospray
Atmospheric pressure photoionization (APPI)	Ionization process	Complement to APCI and ESI

are the unique ionization phenomena: thermospray[1–3] (TSP), electrospray (ESI), and atmospheric pressure chemical ionization (APCI). The reader interested in further detail regarding the historical development of LC/MS, including the impact of techniques such as TSP in pharmaceutical analysis, is referred to the cited references 1–4.

B. Ionization Processes for Modern LC/MS

1. Electrospray

Although TSP and perhaps continuous-flow fast atom bombardment (CF-FAB) had a significant impact on pharmaceutical analysis, the ESI ionization process[5] has created a revolution in the field of mass spectrometry and the application of mass spectrometry to problems in the

pharmaceutical and biological sciences. The ESI process is shown schematically in Figure 2A, and described very succinctly by Grayson,[1] and Norwood et al.,[6] with a detailed discussion of applications presented by Lee.[7] One of the most important features of the ESI process is that ion formation occurs at atmospheric pressure, outside the high vacuum of

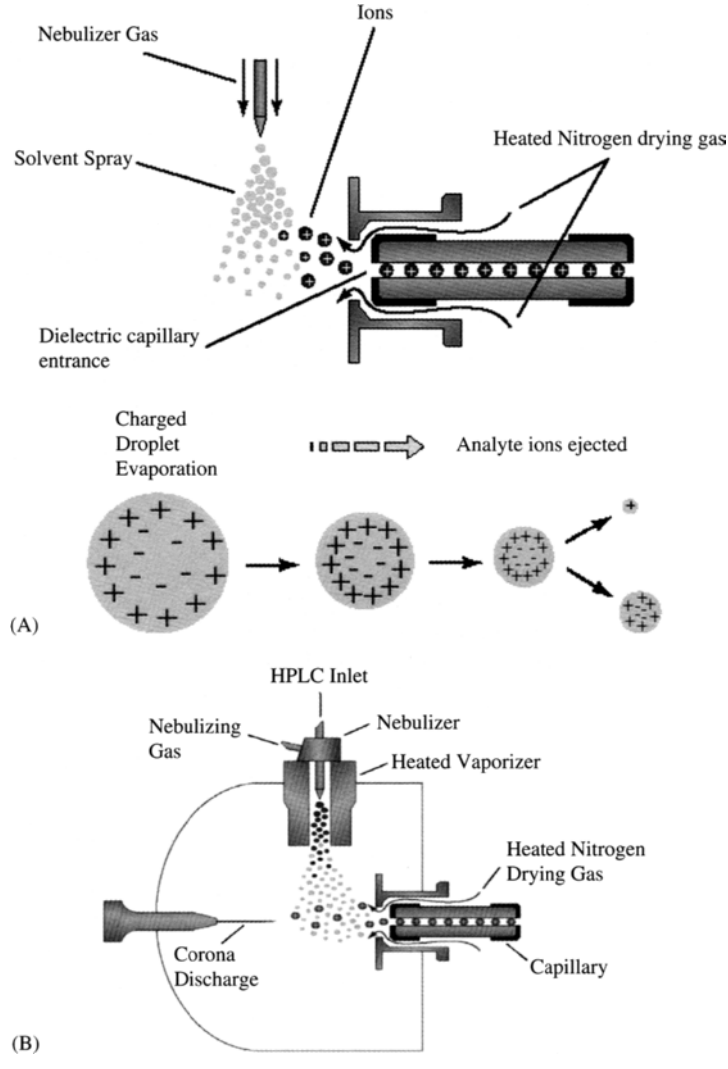


FIGURE 2 (A) Representation of an electrospray ion source and the electrospray process. (Copyright 2005 Agilent Technologies, Inc. Reproduced with permission.) (B) Representation of an atmospheric pressure chemical ionization source. (Copyright 2005 Agilent Technologies, Inc. Reproduced with permission.)

the mass spectrometer. This feature immediately resolves one of the most serious difficulties of LC/MS coupling noted previously, the potential compromise of the mass spectrometer's high vacuum system. Based on the discussions of Grayson,[1] Norwood et al.,[6] and other sources,[8] and with reference to Figure 2A, the ESI process can be simply described. Analyte molecules dissolved in mobile-phase elute from the HPLC column and pass through a tube (stainless steel capillary) with a relatively high positive or negative voltage applied to the tip (e.g., 3–5 kV).[6,8] The resulting electric field and pressure differentials[1] form the eluent into a spray of very small charged droplets, which rapidly evaporate (i.e., desolvate). As evaporation occurs and the droplets reduce in size, the surface charge on the droplets increases until they rupture under the influence of electrostatic forces.[1,6,8] During a succession of such processes, analyte ions are ejected from the charged droplets and are sampled by an orifice into the mass spectrometer for analysis. This sampling orifice is, in most modern ESI sources (see Figure 2A), off-axis from the direction of the spray, which reduces contamination of the orifice and other parts of the mass spectrometer by excess mobile-phase ions and neutral species. Note that this is a rather simplistic description of the ESI process and what actually takes place is somewhat more complex.[9]

The features of ESI which make it ideal for LC/MS, guide its analytical application in pharmaceutical sciences, and impact HPLC method development are:

1. *The ESI process occurs at atmospheric pressure*: This resolves the problem of direct introduction of HPLC column eluent into the mass spectrometer's high vacuum system. Modern ESI sources are designed to reduce contamination of the mass spectrometer, making the ESI-LC/MS technique extremely rugged and robust.
2. *ESI mass spectra tend to reflect solution chemistry:* Analyte ions existing in the charged droplets are ejected and sampled into the mass spectrometer for analysis. Manipulating solution chemistry (i.e., pH, etc.) through the use of volatile buffers (e.g., ammonium formate, ammonium acetate, etc.) can enhance ESI sensitivity. Note that certain mobile-phase additives can suppress ESI sensitivity. For example, significant concentrations of trifluoroacetic acid can suppress negative ion ESI sensitivity.
3. *ESI spectra often include multiply charged ions:* One of the most significant features of ESI for the analysis of biomolecules, such as peptides and proteins, is the production of multiply charged ions (i.e., multiply charged molecular ions) as shown in Figure 3. Since the mass spectrometer separates ions according to their mass-to-charge ratios, the ability to generate such multiply charged ions greatly increases the effective "mass range" of the mass spectrometer, making it useful for the analysis of relatively high molecular

weight biomolecules. Software applications based on sophisticated mathematical techniques are available which can deconvolute such spectra as in Figure 3, yielding the actual molecular weight of the analyte molecule.[1]

4. *ESI is a soft ionization process:* ESI spectra show mostly stabilized even-electron molecular ions (e.g., $[M + H]^+$, $[M + Na]^+$), making it an extremely important process for molecular weight determination.
5. *ESI prefers relatively low mobile-phase flow:* Most ESI-LC/MS systems perform with optimum sensitivity at HPLC flow rates of <0.5 mL/min, with some operating best at μL/min flow rates. This is a general statement, which reflects current technology, and the reader is advised that flow capabilities are a function of source design, which continues to evolve.

2. Atmospheric Pressure Chemical Ionization

APCI is another widely applied LC/MS interface/ionization process in pharmaceutical analysis.[10] Like ESI, APCI works at atmospheric pressure outside the high vacuum of the mass spectrometer. Analyte molecules dissolved in mobile-phase elute from the HPLC column and pass through a heated probe (e.g., 300–600°C), and assisted by an inert nebulization gas (i.e., nitrogen) are vaporized into a fine mist of rapidly evaporating liquid droplets.[1,6] These evaporating droplets and the resulting gas-phase analyte and mobile-phase molecules enter the region of a corona discharge (see Figure 2B). The corona serves to ionize the relatively abundant mobile-phase molecules (e.g., water, methanol, acetonitrile) producing a steady-state population of "reagent gas" ions, which through ion–molecule reactions in the gas phase, ionize analyte molecules. This is, as stated in the name, a Chemical Ionization process.[11]

The features of APCI, which make it ideal for LC/MS, guide its analytical application in pharmaceutical sciences and impact HPLC method development are:

1. *Like ESI, APCI occurs at atmospheric pressure eliminating risk to the mass spectrometer's high vacuum system:* Most ESI sources have the ability to be easily converted to APCI operation with a change in probe and the addition of a corona discharge pin (along with minor changes in gas flows and electronics). This means that an analytical chemist can accomplish both ESI and APCI LC/MS analyses on the same sample with relative ease.
2. *APCI is a gas-phase process, and APCI mass spectra reflect gas-phase ion chemistry (see later discussion).*
3. *Like ESI, APCI is a soft ionization process.*
4. *Unlike ESI, most APCI sources perform best at flow rates of 0.5–1.5 mL/min:* This suggests that APCI might be a good interface

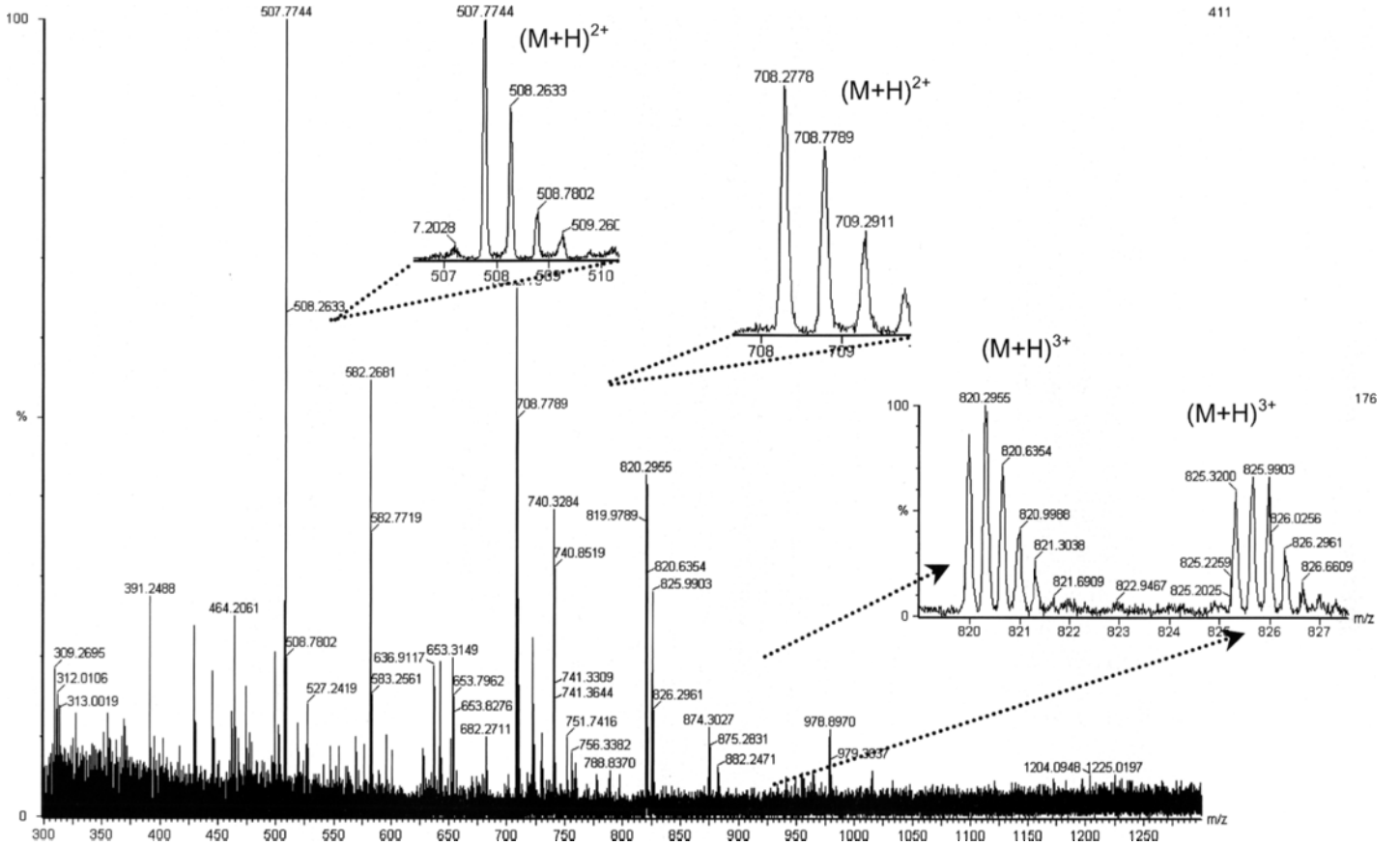


FIGURE 3 Electrospray mass spectrum of a peptide showing multiply charged ions. (Courtesy of Dr. Vladimir Papov, Boehringer Ingelheim Pharmaceuticals, Inc.)

for "analytical scale" HPLC. (The reader is advised, however, that LC/MS source design continues to advance. The future will likely see low flow rate APCI sources, combination ESI/APCI sources for simultaneous acquisition, etc.)

3. Atmospheric Pressure Photoionization

Atmospheric pressure photoionization (APPI) is a relatively new LC/MS ionization process, which one can consider as an evolution in APCI.[12,13] APPI has been shown to be particularly effective at ionizing certain weakly polar analytes. The APPI process typically employs an APCI probe (heated as with APCI) to pass analyte molecules dissolved in HPLC mobile phase into the vicinity of a gas discharge lamp generating vacuum-ultraviolet photons at 10 and 10.6 eV.[14] Photons of these energies can ionize most analyte molecules but are insufficient to ionize the most commonly used HPLC mobile-phase solvents[14] such as water (12.6 eV) and acetonitrile (12.2 eV). This can result in significant reductions in the chemical background of mobile-phase ions commonly observed in ESI and APCI experiments. The application of APPI-LC/MS in pharmaceutical analysis is increasing.[15]

4. Other Ionization Processes

Desorption and surface ionization processes have been used for LC/MS, the first and most widely applied being CF-FAB.[16] More recently, the technique of matrix-assisted laser desorption ionization (MALDI)[17] has been used as an LC/MS ionization process. MALDI, as the name implies, uses an organic matrix designed to absorb laser energy, which is then transferred to the analyte molecules dispersed in the crystalline matrix.[1] The solid matrix is created by mixing analyte molecules with matrix molecules in a solution, and then allowing the solution to evaporate as a spot on a target surface for the laser. MALDI has been shown capable of ionizing very large biomolecules, with molecular weights of >100,000 Da.[1] MALDI has been employed as an ionization process for on-line LC/MS using either a continuous-flow probe or aerosol spray.[18,19]

C. Mass Analyzers

As noted in Figure 1 and Table 1, there are a number of mass analysis systems which have been employed on various LC/MS instruments. The vast majority of LC/MS systems currently in use for pharmaceutical analysis are based on quadrupole mass filters, quadrupole ion traps, TOF, and ion cyclotron resonance (ICR).

The quadrupole mass filter,[20] as illustrated by the schematic of a triple-quadrupole mass spectrometer shown in Figure 4, is a physically

simple but theoretically complex device. The quadrupole separates ions according to their mass-to-charge ratios (m/z) by employing oscillating dc and radio frequency (rf) electric fields. Ions of particular m/z follow a stable trajectory through the filter and onto a detector under particular electric field conditions, while ions of different m/z values have unstable trajectories under the same electric field conditions and are not transmitted. The quadrupole was recognized early on as being a good match with chromatographic inlet systems because, unlike early magnetic sector mass spectrometers it could be scanned quickly, allowing several mass spectra to be acquired across an eluting chromatographic peak. Also, since the quadrupole is a linear scanning device, it is much simpler to calibrate the mass scale and change mass range scanning conditions without the requirement for recalibration.

The triple quadrupole mass spectrometer shown in Figure 4 is capable of accomplishing so-called tandem mass spectrometry (or MS/MS) experiments which is a great advantage for LC/MS.[4] In this experiment, the first quadrupole is set to transmit only one particular ion of interest (e.g., the $[M + H]^+$ of an eluting unknown in an APCI-LC/MS experiment). The second quadrupole is in "rf only" mode, meaning that it will focus and transmit all ions without any m/z separation. The second quadrupole is filled with an inert gas such as argon and acts as a collision chamber, in which the ion of interest transmitted by the first quadrupole collides with argon atoms, gains internal energy in its chemical bonds, and fragments as a result of the excess internal energy. The third quadrupole is then scanned over an appropriate mass range resulting in a collision-induced dissociation (CID)

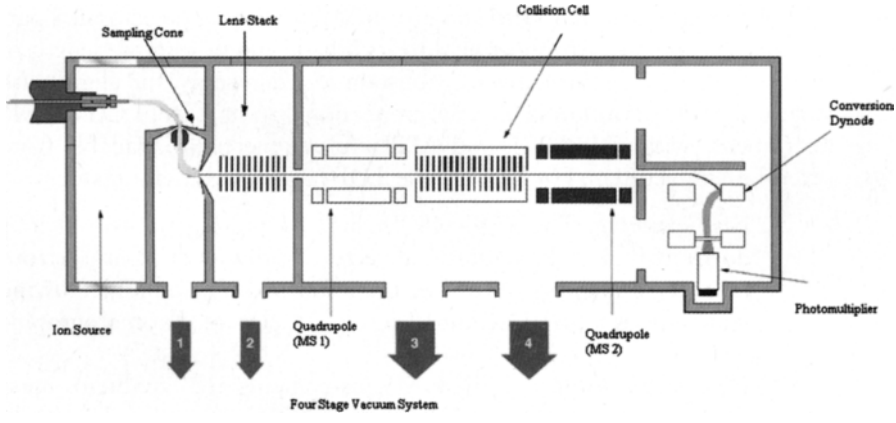


FIGURE 4 Schematic diagram of a triple quadrupole mass spectrometer. (Reproduced with permission from Waters, Inc.)

mass spectrum of the original ion of interest. MS/MS experiments are obviously useful for structure elucidation and are also commonly employed for high sensitivity quantitation with LC/MS (see later discussion).

A cubic quadrupolar field can be used to trap ions and selectively eject them to a detector.[1] Such an instrument is called (appropriately) a "quadrupole ion trap" mass spectrometer. Ions of interest (e.g., the $[M + H]^+$ of an eluting unknown in an APCI-LC/MS experiment) can be trapped, subjected to CID, and the resulting fragment ions mass analyzed and detected. Additionally, a fragment ion from the original ion of interest could be trapped, fragmented, and the resulting secondary fragment ions mass analyzed and detected. This process can continue as long as sufficient numbers of ions are available, and is referred to as an MS^n experiment, where n is the number of successive MS/MS experiments. Such a process is capable of producing fragmentation maps of unknown analyte molecules, where one fragment ion is structurally linked to its precursor and then to the previous precursor, and so on. This is a powerful process for structure elucidation.

The TOF mass spectrometer was first introduced in the 1950s,[1] but has become useful for LC/MS only recently (i.e., late 1990s to the present). A typical TOF-LC/MS instrument is shown schematically in Figure 5. The function of this instrument can be described by the following equation:[4]

$$T = \frac{Lm^{1/2}}{(2zV)^{1/2}}$$

where T is the flight time of an ion; L, length of the flight path for the ion; m, mass of the ion; z, charge on the ion; and V, kinetic energy (accelerating voltage) of the ion.

From this equation, the m/z of any ion arriving at the detector at a particular time is directly proportional to its kinetic energy and the length of the flight path. Therefore, given a constant kinetic energy and flight path length, the m/z of an ion is directly proportional to its TOF. LC/TOF-MS instruments with ESI, APCI, and APPI are commercially available from several manufacturers. For LC/MS, the TOF has several useful features:

1. *Fast scanning:* The TOF-MS "scans" by pulsing packets of ions down its flight tube toward a detector. With modern fast electronics and computers, this can be accomplished very rapidly allowing many spectra to be acquired across an eluting chromatographic peak.
2. *High mass resolution:* TOF-MS instruments are capable of mass resolving powers of approximately 5000 (50% valley definition) or greater, allowing for increased specificity and selectivity over low resolution instruments (e.g., quadrupoles). This is facilitated

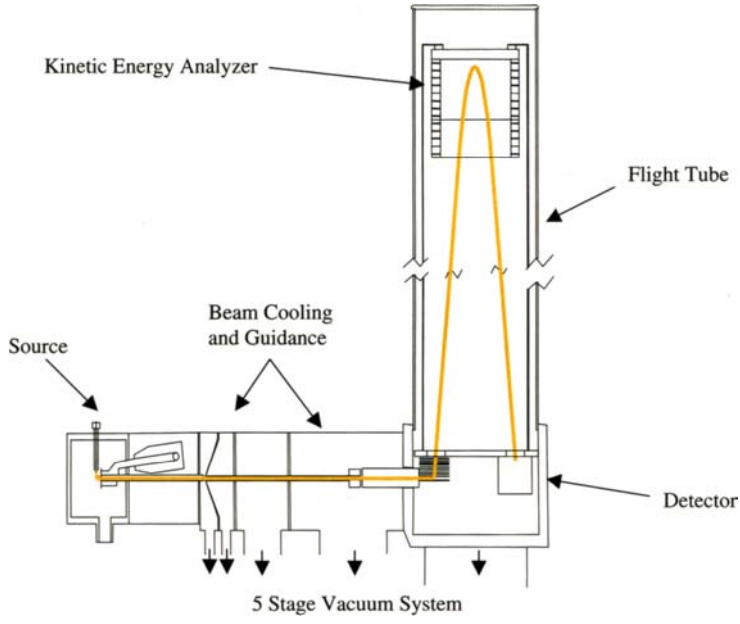


FIGURE 5 **Schematic diagram of a time-of-flight mass spectrometer. (Copyright 2005 Agilent Technologies, Inc. Reproduced with permission.)**

by kinetic energy filters, which remove metastable ion transitions that can cause ion beam broadening.

3. *Accurate mass measurements:* Given careful control over V and L, TOF-MS instruments are capable of measuring m/z values to <5 ppm accuracy, allowing elemental compositions of ions to be determined.

Also, available for the past several decades is the ICR mass spectrometer.[1] This instrument operates by measuring the cyclotron resonance frequency of an ion, which is directly proportional to its m/z as follows:[4]

$$m/z = \frac{B}{2f_c}$$

where B is the laboratory magnetic field strength and f_c, cyclotron resonance frequency.

The laboratory magnetic field (B) is produced by a superconducting magnet with a "cyclotron cell" capable of trapping ions injected into it (not unlike a quadrupole ion trap).[1] Trapped ions are excited by application of an rf voltage within the cell, take on a radial motion away from the central axis of the cell, and follow a circular path.[21] The resulting cyclotron resonance frequencies of all ions (whatever their m/z) within

the cell are recorded as an "image current", the individual frequencies are extracted via Fourier-transform (the ICR is commonly referred to as an FTMS or Fourier-transform mass spectrometer), and the results presented as a normal mass spectrum (% relative abundance versus *m/z*).[21] Ionization (ESI, etc.) occurs outside the cell and the ions are sent into the cell and trapped. For LC/MS, the ICR (FTMS) has several useful features:

1. *Ultra-high mass resolution:* The FTMS is capable of mass resolving powers of >500,000 (50% valley definition).
2. *High mass accuracy:* The FTMS is easily capable of 2 ppm mass accuracy or better depending on whether external calibration or internal reference ions are used.
3. *MS^n:* When combined with other mass spectrometers (such as the tandem quadrupoles or ion traps) to form hybrid instruments, the FTMS is capable of MS/MS and/or MS^n experiments, but with high mass accuracy. Such an instrument (a linear quadrupole ion trap combined with an FTMS) is shown in Figure 6. The potential of such hybrid instruments for structure elucidation is enormous, linking precursor–fragment relationships with elemental compositions of all precursor and fragment ions obtained from the FTMS.

Note that other hybrid instrument configurations are also possible. Perhaps, the most popular of which is the combination of the quadrupole with TOF (see Figure 7). In this instrumental configuration, a resolving quadrupole selects ions of interest, which are collisionally dissociated in a second quadrupole (in this case a hexapole), with the resulting fragment ions mass analyzed by the TOF. The advantage is higher mass resolution and mass accuracy for the collision-induced fragment ions.

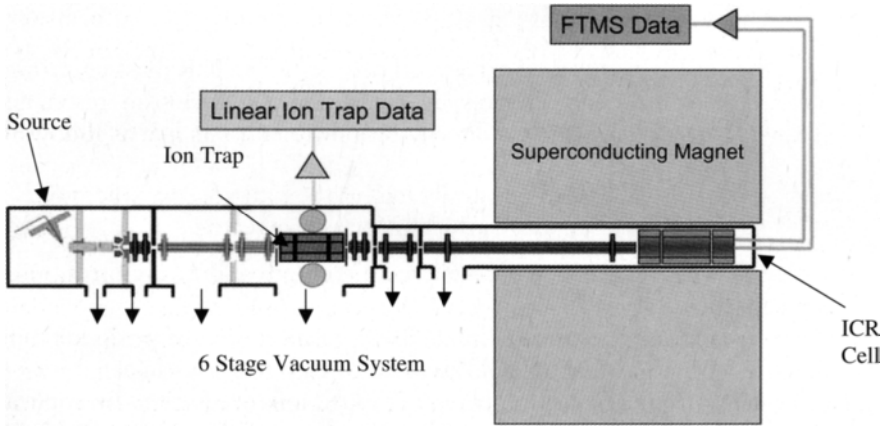


FIGURE 6 Schematic diagram of a hybrid linear ion trap FTMS, the LTQ-FT. (Reproduced with permission from Thermo Electron Corporation.)

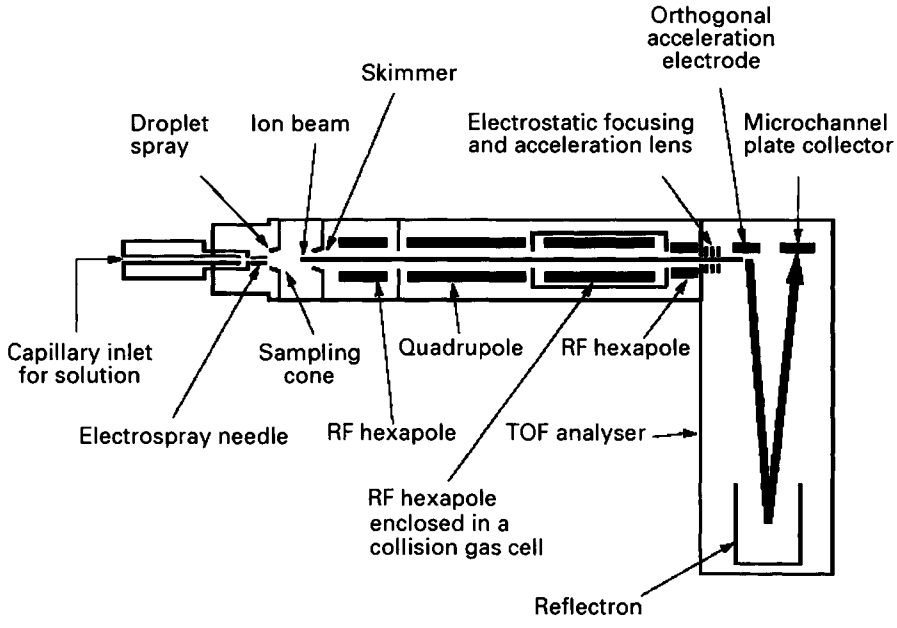


FIGURE 7 Schematic diagram of a hybrid quadrupole/time-of-flight mass spectrometer, the Q-TOF. (Reproduced with permission from Waters, Inc.)

Instrumental advances continue for LC/MS, with evolutions in ion source design, ionization processes, and mass analysis.

D. Chromatography Method Development for LC/MS: General Considerations

The types of chromatography separation systems and platforms used with mass spectrometry in the pharmaceutical industry, and the method development implications for these are dependent on:

1. The nature of each individual problem within a particular application area.
2. The constraints and parameters of the individual application.
3. The use of the mass spectrometer as a detector.

The type of LC/MS system and particular ionization process applied to a particular problem are also application and problem dependent. Pharmaceutical applications employing LC/MS can be generally grouped into those supporting drug discovery efforts (e.g., genomics, proteomics, natural product dereplication, combinatorial chemistry/library generation, and high-throughput screening applications), ADME studies (including metabolite identification), DMPK quantitative bioanalysis, pharmaceutical development projects and regulatory submissions

(e.g., impurity and degradant profiling, container closure system and packaging issues, etc.), and manufacturing and quality control. These application areas for LC/MS have been comprehensively reviewed by Lee,[7] Papac and Shahrokh,[22] Pramanik et al.,[23] Cristoni and Bernardi,[24] Zhou et al.,[25] Oliveira and Watson,[26] Niessen,[27] Berna et al.,[28] Wolfender et al.,[29] and Hopfgartner and Bourgogne.[30] It is also important to highlight the comprehensive book on problem solving with LC/MS by Willoughby et al.,[31] as well as the contribution of Cohen and Rossi.[32] The pharmaceutical problems to which LC/MS is applied can be grouped into the two general areas of qualitative and quantitative. Qualitative problems include: identification of drug substance process impurities and degradation products, metabolite identification, bioorganic macromolecule characterization, and high-throughput screening applications. The high sensitivity of LC/MS combined with the significant structure elucidation information available from mass spectra are central in addressing qualitative problems. Quantitative problems include: DMPK quantitative bioanalysis for pharmacokinetics and other studies (where LC/MS is now considered the primary analytical technique), assay of animal feed materials in support of toxicology studies, and mass balance studies. The high sensitivity of LC/MS and the selectivity of the mass spectrometer relative to other types of HPLC detectors are central for quantitative problem solving.

As stated above, each application area has its own individual constraints and parameters, which affect the HPLC systems and platforms employed, and the LC/MS systems and ionization processes applied. For example, an analyst responsible for identifying process impurities is usually presented with a chromatogram with UV detection containing trace level unknown peaks obtained using an approved regulatory HPLC method. This HPLC method may not be LC/MS compatible (i.e., it may contain involatile mobile-phase additives, see below), and is most often based on "analytical" HPLC (e.g., 4.6 mm i.d. columns and 1 mL/min flow rate). If not LC/MS compatible, the HPLC method would need to be redeveloped with volatile buffers, and the unknown peaks correlated between the two methods. The parameters of analytical scale HPLC are a good match for APCI (ESI would require flow splitting for best performance on most instruments). Unlike process impurities whose detection is constrained by regulatory HPLC methods, unknown metabolites often present themselves to the mass spectrometrist from a variety of HPLC method types, especially the increasingly popular "μ-bore" (e.g., 2.1 mm i.d., 5 cm length, 200 μL/min flow rate). These HPLC platforms are on the edge of compatibility with most APCI sources (0.2 mL/min is low for many APCI sources), but very much in the optimum range for ESI. A researcher in bioorganic macromolecules (e.g., biomarkers) might employ capillary HPLC (e.g., 0.5 mm i.d., 5 μL/min flow rate) or even "nanoscale" HPLC (e.g., 50–100 μm i.d., 20–500 nL/min flow

rate). These HPLC platforms interface best with ESI, with nanoscale chromatography requiring specially designed ESI sources. High-throughput application areas often use flow-injection LC/MS (i.e., without an HPLC column for separation), and newer instrument designs have special ESI sources which can simultaneously acquire data from multiple (i.e., 4–6) flow-injection or HPLC systems.

The review articles cited in this chapter list and describe the various pharmaceutical application areas for LC/MS, and discuss many of the typical HPLC platforms employed for each application area. The bottom line is that there is no major application area in either drug discovery or pharmaceutical development in which LC/MS has not had a significant impact. There are no fundamental constraints or limitations presented to the application of LC/MS by any of the most popular HPLC techniques and platforms used in any of the application areas.

So, what issues do chromatographers need to consider when developing methods for LC/MS, or methods, which might require LC/MS at some future time? To answer this question, it is useful to ask and address another:

> If the mass spectrometrist was in charge of the HPLC method development process, what guidelines would be applied?

1. *The mass spectrometrist would first consider the application area and the problem.* The more regulated the application area is, the more constrained are the analytical methods. Manufacturing, quality control, and pharmaceutical development areas are the most regulated (by current good manufacturing practices (cGMP)) and, therefore, rely on validated and approved chromatographic methods. If a given method is not LC/MS compatible then, as previously stated, it would need to be carefully redeveloped, the redevelopment documented, and unknown impurity peaks correlated back to the approved chromatographic method. Quantitative LC/MS methods developed in support of toxicology or clinical studies (i.e., good laboratory practices (GLP) regulated studies) would need to be fully validated according to accepted parameters.[33] In non-regulated application areas, mass spectrometrists would prefer short run times and simple mobile-phase conditions. The selectivity of the mass spectrometer as a detector means that it is not necessary to separate all analytes, as a chromatographer with a poorly selective UV detector would need to do.
2. *The mass spectrometrist would choose the most appropriate ionization process to solve a given problem.* Problems involving small molecule drugs can be addressed by either ESI or APCI. If the chromatographic method is constrained to analytical scale

(see above) then APCI is often the best choice. If chromatographic methods with lower flow rates are chosen (μ-bore or nanoscale), then ESI is the best choice. With bioorganic macromolecules, ESI is always the ionization process of choice (unless HPLC-MALDI is available and required because of the high molecular weight of the analyte).

It is important to evaluate the molecular structure of the proposed analyte (in the case of quantitative LC/MS method development), or the parent drug (in the case of unknown impurity or metabolite identification). One must consider proton affinity and the possibilities for proton abstraction. Analytes with reduced nitrogen atoms in their structure (e.g., primary and secondary amines) have generally high proton affinity and will likely form stable $[M + H]^+$ ions in the gas phase. Carboxylic and sulfonic acids easily abstract protons to form stable $[M - H]^-$ ions making negative ion APCI or ESI attractive alternatives. For ESI, one must always consider if the analyte molecule is likely to be (or can be forced to be) ionized in solution. For certain types of molecules (e.g., nitro-compounds), which have neither high proton affinity nor easy proton abstraction, alternatives are available (see the later discussion regarding gas-phase ion chemistry).

3. *The mass spectrometrist would prefer reverse-phase HPLC methods and platforms.* Reverse-phase solvents (water, methanol, acetonitrile, etc.) perform well in either ESI or APCI (also APPI). It is possible to use normal-phase solvents,[32] but to a mass spectrometrist, this is not desirable. Tetrahyrdofuran (THF) should be avoided because of safety issues and its effects on HPLC tubing and seals;[32] however, in our experience, THF can be used in a limited way if required for elution of very non-polar analytes.
4. *The mass spectrometrist would choose HPLC method parameters that are most compatible with the chosen ionization process and that take advantage of the selectivity and sensitivity of the mass spectrometer.* These include: short run times, simple mobile-phase gradients, flow rates compatible with the ionization process of choice, and as few mobile-phase additives as possible.
5. *The mass spectrometrist would always employ LC/MS compatible mobile-phase additives.* In the mass spectrometrist's point of view, chromatographers rely entirely too much on inorganic buffers and ion-pairing reagents. The primary offenders are inorganic salts of phosphate, perchlorate, and inorganic ammonium and sulfonic acid salts. There are many alternative buffers (e.g., ammonium acetate, ammonium formate, acetic acid, formic acid, trifluoroacetic acid, etc.)[31,32] and ion-pairing reagents (e.g.,

heptafluorobutyric acid) available. For additional information and choices, the reader is referred to the previously cited references on buffers, as well as Duchateau et al.,[34] Vervoort et al.,[35] de Miguel et al.,[36] Franey,[37] Zacharis et al.,[38] and Fischer et al.[39] Although there have been reports of involatile mobile-phase additives (e.g., sodium phosphate and sodium octanesulfonate[40]) being used with certain atmospheric pressure sources, this practice is not generally recommended.

It is important to note that certain volatile mobile-phase additives can cause analyte ionization suppression with resulting sensitivity loss.[31,32] This has been particularly noted with trifluoroacetic acid and peptide analytes,[41] and would likely also be true of other perfluorinated acids especially in negative ion mode. Caution should be exercised when using relatively strong volatile acids and quaternary ammonium compounds as mobile-phase additives. The interested reader is also referred to the work of Choi et al.[42] and Mallet et al.[43]

6. *The mass spectrometrist would consider how to manipulate the HPLC system to improve LC/MS detection and enhance information content in the mass spectra.* Since ESI relies on solution chemistry, the mass spectrometrist would consider how to form ions in solution. The most obvious way is to manipulate mobile-phase pH either up or down. The mobile-phase traveling through the HPLC column need not be manipulated, since additions can be accomplished post-column with appropriate equipment and plumbing. In APCI, it has been reported that methanol can enhance sensitivity relative to acetonitrile.[32] This is related to the properties of the two solvents when acting as CI reagent gasses in APCI-LC/MS. Two additional and unique mobile-phase manipulations are discussed below.

E. Qualitative Analysis by LC/MS

Although NMR may be a more definitive analytical technique for organic structure elucidation (see the following discussion), mass spectrometry is unsurpassed for the qualitative analysis of complex mixtures of trace level organic chemical entities. The mass spectrometer is a "compound specific" detector,[44] providing information directly related to the molecular structure of an organic chemical analyte. The information from mass spectrometry includes:

1. *Molecular weight:* The molecular weight of an unknown is the single most important piece of information available from mass spectrometry. This critical data cannot be obtained from other analytical techniques, including NMR. Figure 8 shows in-line UV

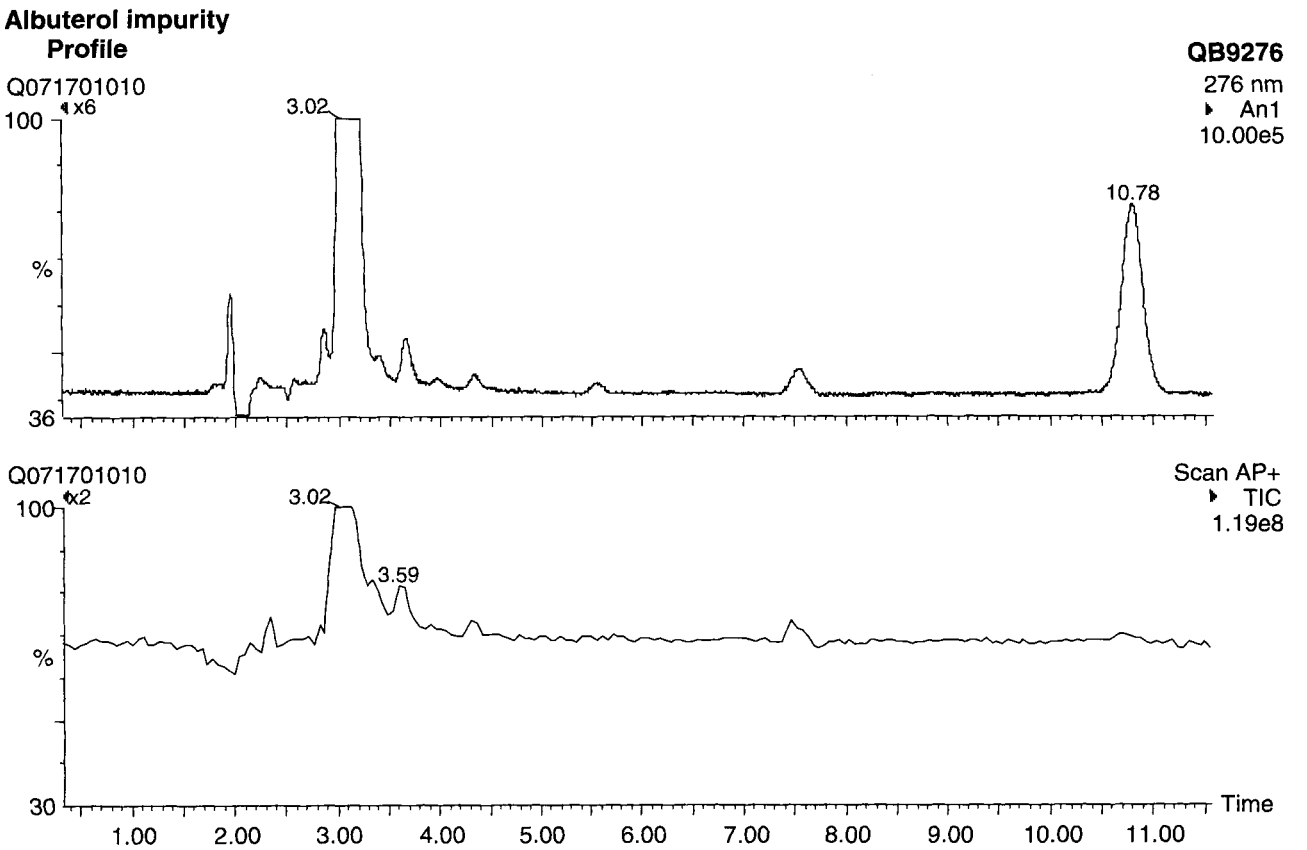
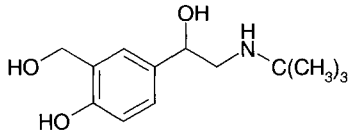


FIGURE 8 In-line UV (276 nm) chromatogram (top) and total ion chromatogram (bottom) from the APCI LC/MS analysis of Albuterol drug substance; note that the mobile phase contained heptafluorobutyric acid as an ion-pairing reagent.

(276 nm) and total ion chromatograms (TIC) from a positive ion APCI LC/MS analysis of Albuterol drug substance:



Note that while the UV chromatogram shows a number of trace level impurity peaks, the TIC shows very little. This is not a manifestation of poor LC/MS sensitivity, but results from the significant chemical background often observed in LC/MS from mobile-phase ions. Impurity peaks are observed by plotting extracted ion current profiles (also called mass chromatograms) as in Figure 9. The positive ion APCI spectrum of Albuterol and two of the trace level impurities are shown in Figure 10. One impurity has a molecular weight of 253 ($[M + H]^+$ at *m/z* 254) suggesting the addition of a methyl group to the Albuterol molecule (MW 239; $[M + H]^+$ at *m/z* 240), and the second impurity has a molecular weight of 257 ($[M + H]^+$ at *m/z* 258) suggesting the addition of water. This is a significant amount of structural information derived from this one simple LC/MS experiment.

Adduct ions are useful for confirming molecular weights. Consider the positive ion APCI spectrum of a trace level drug substance impurity shown in Figure 11, with ions at *m/z* 619 and 636 confirmed to be associated with the unknown structure. Ion *m/z* 636 is 17 mass units higher than 619, suggesting that it is an $[M + NH_4]^+$ and, therefore, *m/z* 619 is the $[M + H]^+$, which confirms the molecular weight of the unknown to be 618. It was also helpful to be aware that the HPLC mobile phase in this LC/MS analysis contained ammonium acetate. There are many commonly observed adduct ions in positive ion APCI and ESI, including $[M + NH_4]^+$, $[M + Na]^+$, $[M + K]^+$, $[M + H + \text{acetonitrile}]^+$, $[2M + H]^+$, $[2M + Na]^+$, etc. The analyst must always be aware of what in the mobile phase could generate unique adduct ions, which could then be used for molecular weight confirmation. In negative ion ESI and APCI, it is common to observe $[M - H]^-$ along with adducts such as $[M + \text{trifluoroacetate}]^-$ and $[M + Cl]^-$.

2. *Molecular formula:* The elements in the periodic table are composed of stable isotopes (e.g., $^{12}C/^{13}C$, $^{14}N/^{15}N$, $^{35}Cl/^{37}Cl$, etc.) with different natural abundances and unique accurate masses. The mass spectrometer is capable of separating these isotopes for molecular and fragment ions, and certain mass spectrometers (i.e., magnetic sector, TOF, FTMS) are capable of mass measurements of sufficient accuracy (i.e., 3 ppm or less for molecules of approximately 700 molecular weight and less) so as to allow special computer programs to suggest the elemental composition of the ion. If the ion is an $[M + H]^+$, for example, then the molecular formula

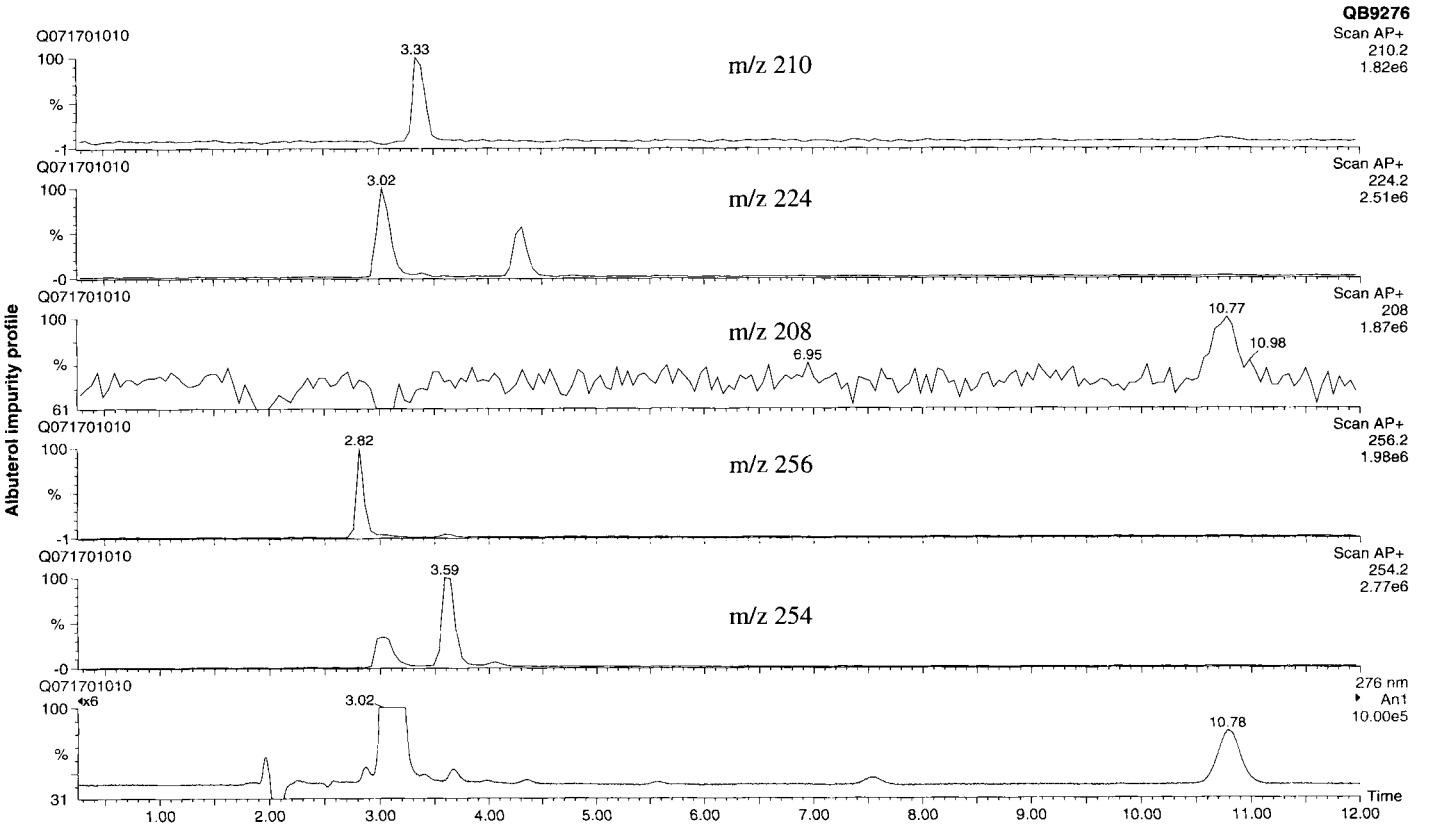


FIGURE 9 In-line UV (276 nm) chromatogram (bottom) and various mass chromatograms (APCI-LC/MS) locating Albuterol drug substance impurities.

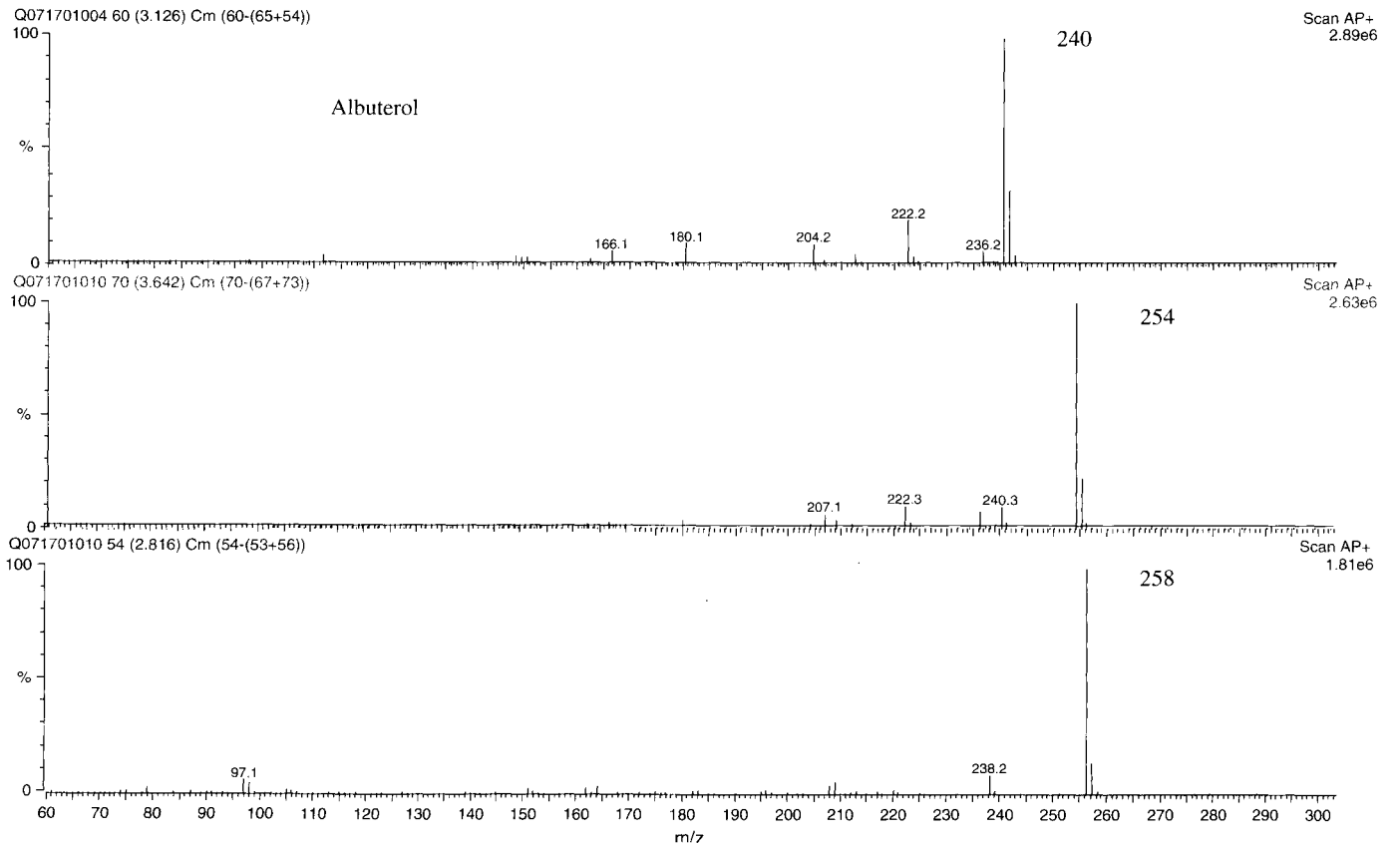


FIGURE 10 **APCI mass spectra of Albuterol drug substance (top) and two of the located impurities with $[M + H]^+$ ions labeled.**

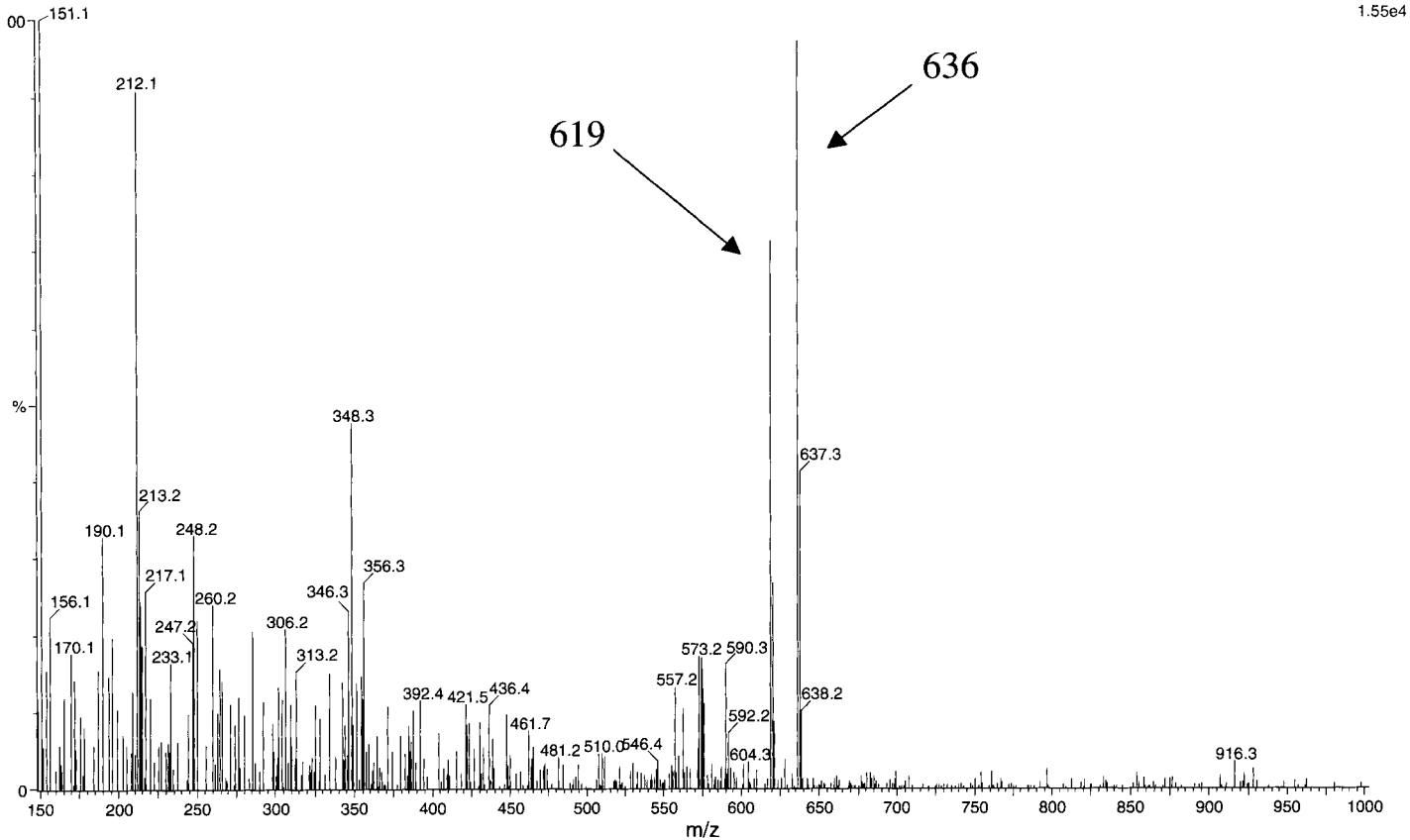


FIGURE 11 **APCI mass spectrum of an unknown drug substance process impurity.**

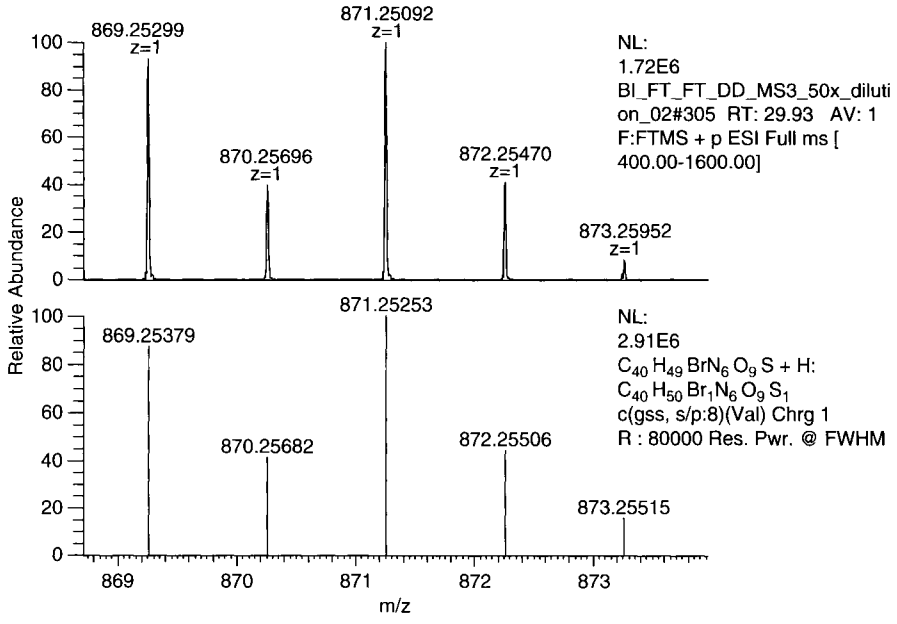


FIGURE 12 ESI mass spectrum of a drug substance (molecular ion region only; [M + H]$^+$) acquired on an LTQ-FT (hybrid linear ion trap/FTMS).

of the unknown can be determined. Figure 12 shows the accurate mass measured $[M + H]^+$ from an ESI LC/MS analysis on an FTMS instrument. Note that the accurate mass measurement confirms the known molecular formula of $C_{40}H_{49}BrN_6O_9S$ (0.920 ppm accuracy on the first isotope peak). Instruments such as the QTOF and hybrid FTMS (e.g., the LTQFT in Figure 6) are capable of accurate mass measurements on product ions associated with a mass selected parent such as a molecular ion.

3. *Fragmentation behavior:* The gas-phase chemical reactions, which generate fragment ions from molecular ions and from other higher mass fragment ions are well understood from fundamental principles.[45,46] It is, therefore, possible to propose a molecular structure for an unknown from its observed fragmentation behavior. Fragmentation of the molecular ion can occur either in the LC/MS ion source or via CID in a triple quadrupole, QTOF, ion trap, or FTMS. The ion trap and FTMS are capable of trapping selected ions and subjecting them to further CID, to yield further fragment ions in so-called MS^n processes.[4,6] The QTOF and FTMS are capable of accurate mass measured MS/MS and MS^n, respectively. It is, therefore, possible with the most modern LC/MS instrumentation to construct fragmentation maps with elemental compositions for molecular and fragment ions and establish precursor-product relationships between ions.

TABLE 3 Summary of LC/MS Instruments Generally Applied to Qualitative Pharmaceutical Analysis (When Interfaced with ESI, APCI, APPI)

LC/MS instrument	Information available
Single quadrupole	Molecular weight
	In-source fragmentation
Triple quadrupole	Molecular weight
	In-source fragmentation
	MS/MS
Quadrupole ion trap (cubic or linear)	Molecular weight
	In-source fragmentation
	MS^n
Time-of-flight	Molecular weight
	In-source fragmentation
	Accurate mass measurements
FTMS	Molecular weight
	In-source fragmentation
	Accurate mass measurements
Hybrid QTOF	Molecular weight
	In-source fragmentation
	MS/MS
	Accurate mass measurements (MS and MS/MS)
Hybrid trap/FTMS	Molecular weight
	In-source fragmentation
	MS^n
	Accurate mass measurements (MS and MS^n)
Hybrid quadrupole/FTMS	Molecular weight
	In-source fragmentation
	MS/MS
	Accurate mass measurements (MS and MS/MS)

Table 3 lists the most commonly used LC/MS instruments along with the data, each of which is capable of producing for structure elucidation purposes.

F. Quantitative Analysis by LC/MS

LC/MS is one of the most sensitive and accurate quantitative analysis techniques available for trace organic analysis, and is widely applied in the pharmaceutical industry. LC/MS, with both ESI and APCI, currently has a central role in quantitative bioanalysis where quantitation limits in the pg/mL range for drugs and metabolites in biological fluids are often required. LC/MS quantitation is best accomplished either by selected ion

monitoring (SIM) or selected reaction monitoring (SRM). In an SIM LC/MS experiment, the mass spectrometer is set up to only observe particular ions of interest, for example, $[M + H]^+$ ions from a parent drug and its metabolites. By only observing ions of interest and not wasting time scanning other *m/z* regions, the SIM LC/MS experiment gains the so-called "time advantage", and with it increased sensitivity. Further, SIM experiments collect a significantly increased number of data points across an eluting HPLC peak compared with "full scan" acquisition modes and can, therefore, define the peak with greater precision and accuracy. SIM experiments can be accomplished on any of the common types of mass spectrometers used for LC/MS; however, quadrupoles and triple quadrupoles tend to show the greatest sensitivity increases.

SRM experiments have even greater selectivity and sensitivity advantages than SIM, and such experiments (generally referred to as LC-MS/MS) have come to dominate quantitative LC/MS in pharmaceutical applications. By far, the most utilized instrument for LC-MS/MS is the triple quadrupole mass spectrometer (see Figure 4). A typical LC-MS/MS experiment would begin by selecting precursor ions of interest ($[M + H]^+$ ions from a parent drug and key metabolites, for example), and the first mass filter would be set to pass only these ions. This is accomplished by switching the mass filter between the selected *m/z* values, not unlike what is done in an SIM experiment. The selected precursor ions then enter the collision chamber where CID processes generate known fragment ions, which are then passed by the third mass filter (again by switching between preselected *m/z* values) and detected. Consider the positive ion ESI spectrum of ranitidine in Figure 13, and note the $[M + H]^+$ at *m/z* 315. Figure 13 shows the MS/MS spectrum in which *m/z* 315 has been subjected to CID in a triple quadrupole instrument. Each ion in this CID spectrum is a direct product of *m/z* 315 by a single fragmentation process. A quantitative LC-MS/MS experiment could be developed by monitoring and detecting *m/z* 176 from CID of the *m/z* 315 precursor ion. It is possible (and routine) to have LC-MS/MS methods that monitor several, or many, CID processes from a group of target compounds. Such experiments are referred to as multiple reaction monitoring (MRM) experiments.

Zhou et al.[25] have extensively reviewed bioanalytical LC-MS/MS, including discussions of method development, validation and transfer. Cohen and Rossi[32] have also discussed in detail many important aspects and considerations of quantitative LC/MS. In developing HPLC methods for LC-MS/MS experiments, the chromatographer must remember that the detector is a mass spectrometer:

1. *The high selectivity of LC-MS/MS means that it is not necessary to separate all target analytes, as it would be for UV detection.* Many LC-MS/MS methods simply separate the target analytes from the salts and other "junk" in the void volume. Inorganic

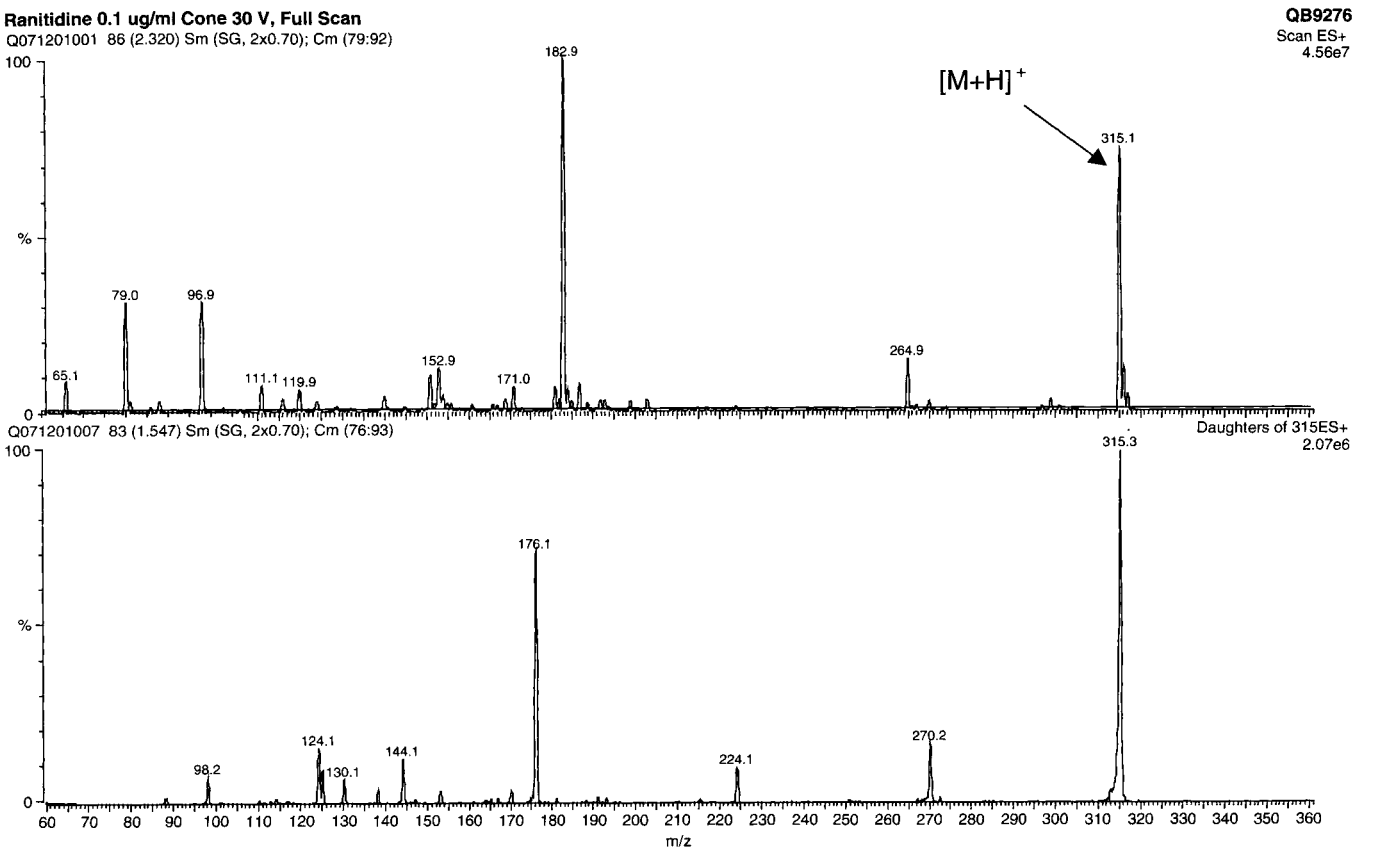


FIGURE 13 ESI mass spectrum of ranitidine drug substance (top) and MS/MS product ion CID spectrum (products of *m/z* 315; the $[M + H]^+$) (bottom).

material can cause ion suppression effects with resulting sensitivity losses and must be separated from target analytes. This means, however, that LC-MS/MS methods can have relatively short run times (a few minutes is typical) and use very short HPLC columns (e.g., 3 cm). ESI and APCI ion sources are also very rugged and robust allowing hundreds of samples to be analyzed without ion source maintenance.

2. *The mass spectrometer is an isotope separator, which means that stable isotope labeled internal standards can be employed with LC-MS/MS.* Internal standards are well known to increase the precision and accuracy of analytical methods, and it is also well established that the closer an internal standard is to an analyte in molecular structure, the greater is the gain in precision and accuracy. There is no closer structural analogue to an analyte than a stable isotopically labeled version of the analyte, with ^{2}H, ^{13}C, and ^{15}N being the most commonly employed. Since they are so close in structure to their target analytes, many stable isotope labeled internal standards cannot be chromatographically separated from the analytes. However, to the mass spectrometer, this is not an issue as the target precursor and product ions would have different *m/z* values. No other quantitative detection system for HPLC can employ internal standards of this type.

G. Ion Chemistry and LC/MS: Analytical Applications

LC/MS experiments rely on chemical reactions to form ions, either in solution as with ESI or in the gas phase as with APCI. This fact can be taken advantage of and manipulated to generate additional structural information, increase sensitivity, and increase ionizability of analytes. There are two notable examples: hydrogen/deuterium (H/D) exchange reactions and negative ion attachment.

The purpose of H/D exchange is to determine the number of active (or exchangeable) hydrogens in an unknown analyte.[47] Exchangeable hydrogens are present in functional groups such as COOH, OH, NH_2, NHR, SH, etc. The determination is accomplished by substituting deuterated solvents for protonated solvents in the HPLC mobile phase. Each active hydrogen, equivalent to 1 atomic mass unit (a.m.u.), is exchanged for a deuterium equivalent to 2 a.m.u. The number of active hydrogens in an unknown analyte is easily determined by comparing the ESI or APCI spectra obtained in deuterated solvents to those obtained in protonated solvents.

Negative ion attachment can enhance the ionizability of "difficult" analytes. For example, consider nitroglycerin:[48,49]

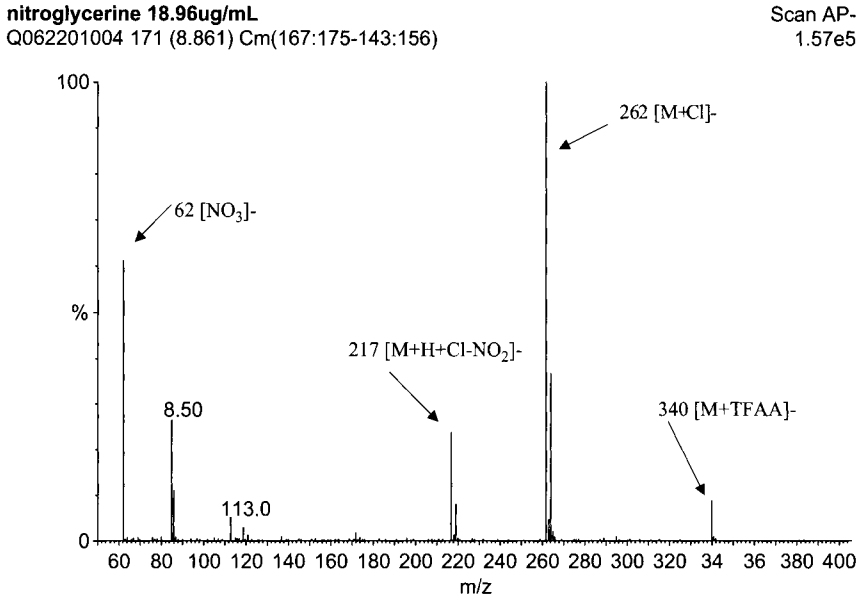
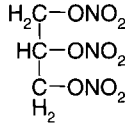


FIGURE 14 **Negative ion APCI chloride ion attachment mass spectrum of nitroglycerin. (Reproduced with permission from American Pharmaceutical Review.)**



This molecule has both relatively poor proton affinity and a lack of potential to form a stable proton abstracted negative ion. However, if a relatively small amount of chloroacetonitrile (1%) is added to the HPLC mobile phase in a negative ion APCI LC/MS experiment, the resulting corona-induced Cl^- can "attach" to the nitroglycerin molecule in the gas phase forming an $[M + Cl]^-$ molecular ion (see Figure 14). Note that the stable isotope pattern of the chlorine is apparent ($^{35}Cl/^{37}Cl$). This technique has been employed to characterize trace level impurities in nitroglycerin.[48,49]

III. COMBINED LIQUID CHROMATOGRAPHY/NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

NMR is one of, if not the most, powerful tools available to pharmaceutical research for the structure elucidation of organic chemical entities. The combination of HPLC with NMR (LC/NMR) offers the potential for

a quantum leap in the problem-solving capabilities of pharmaceutical analysis laboratories, in both drug discovery and pharmaceutical development application areas. The following sections of this chapter describe NMR and LC/NMR, and discuss the implications for HPLC method development of using NMR as a detection system. The use of LC/NMR for pharmaceutical problem solving applications is also critically evaluated from a cost–benefit analysis perspective.

A. The NMR Phenomenon

The phenomenon of NMR is well understood and is described in detail in numerous textbooks and treatices.[50] At an empirical level, NMR can be explained with reference to the coordinate diagram (the so-called "rotating frame" diagram) shown in Figure 15A–C. As illustrated in the figure:

1. Certain atomic nuclei, including the proton (^{1}H) and ^{13}C, possess angular momentum (P), which imparts to these nuclei a magnetic moment (μ).[50] The relationship is described by the following equation:

$$\mu = \gamma P$$

   where γ (termed the magnetogyric ratio) is a constant for a particular atomic nucleus.
2. If the spinning nucleus is placed in a strong magnetic field (B_0, termed the "laboratory field"), its magnetic moment will tend to align in the direction of the field (which is the lower energy state). This is illustrated by the vector along the z-axis in Figure 15A. An ensemble of spinning nuclei will assume a Boltzman distribution aligned with the direction of the magnetic field.
3. If a second magnetic field (B_1) is then placed in the x–y plane along the x-axis at a particular frequency, as illustrated in Figure 15B, the nuclear magnetic moment will attempt to align itself with the lower energy state of that field producing magnetization in the x–y plane.
4. If B_1 is then turned off, μ will return to its former alignment with B_0 by a process(s) termed "relaxation" (Figure 15C). Relaxation releases magnetization in the x–y plane at the particular resonance frequency of the nucleus, which can be detected.

Nuclei of the same type (e.g., protons) in different chemical environments within an organic molecule (e.g., CH_3, CH=CH, aromatic) have slightly different resonance frequencies (termed "chemical shifts"), which can be measured. Chemical shifts for ^{1}H and ^{13}C nuclei of many

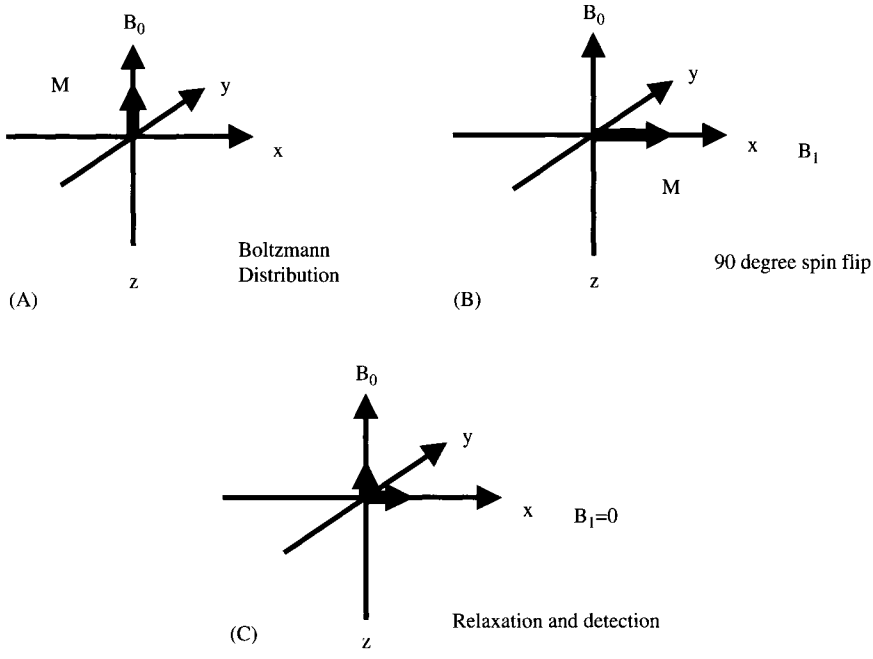


FIGURE 15 (A) Rotating frame representation of a spinning nucleus (z-axis vector) under the influence of a laboratory magnetic field (B_0) along the positive z-axis. (B) Rotating frame representation of a spinning nucleus (z-axis vector) under the influence of a laboratory magnetic field (B_0) along the positive z-axis with a second magnetic field (B_1) added along the positive x-axis. (C) Rotating frame representation of a relaxing spinning nucleus (z-axis vector) under the influence of a laboratory magnetic field (B_0) after B_1 is turned off (note that magnetization is released and detected in the x–y plane).

types and in many different chemical environments have been studied and tabulated.[51] An NMR spectrum in its simplest form (see Figure 16) is a plot of signal intensity versus chemical shift for a given type of nucleus.

B. The NMR Spectrometer

A schematic diagram of an LC/NMR spectrometer is shown in Figure 17. The "laboratory field" (B_0) is created by a cryogenically cooled superconducting magnet. Samples composed of organic chemical analytes dissolved in a solvent are contained in special-purpose glass tubes in a "probe" located inside the superconducting magnet. The probe also contains an rf coil, which creates B_1, and a receiver coil for detecting the released magnetization from relaxing nuclei. This released magnetization is detected and recorded as a sine wave. Since the NMR signal is very weak, modern spectrometers irradiate all nuclei of a particular type (e.g., ^{1}Hs in all chemical environments) at the same

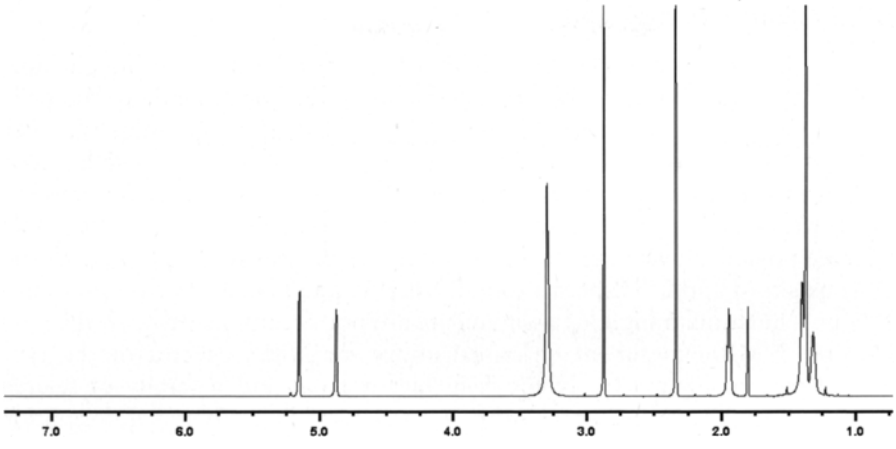


FIGURE 16 A typical 1D ^{1}H-NMR spectrum (chemical shift on the *x*-axis versus intensity).

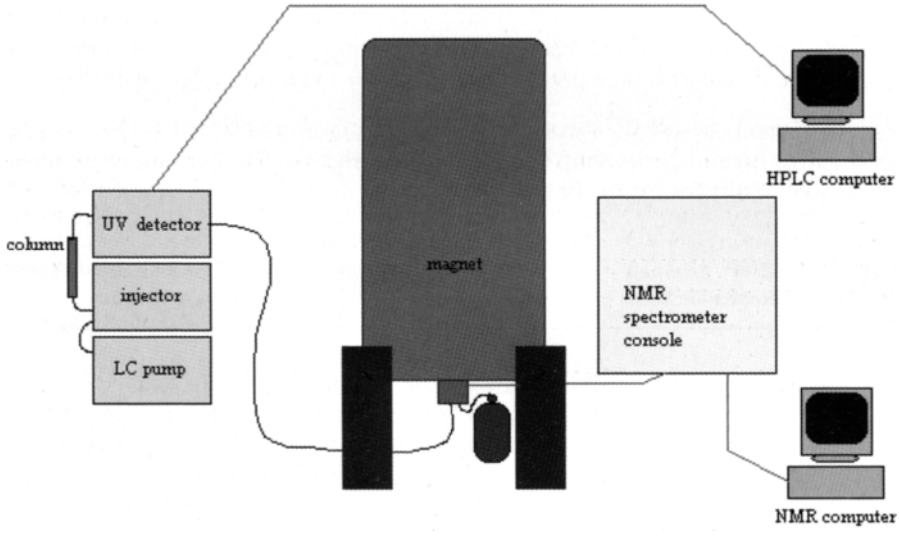


FIGURE 17 Schematic diagram of an LC/NMR spectrometer.

time with an rf pulse (B_1) and then collect all released magnetization signals at the same time in the form of an interfering sine-wave pattern, termed a free induction decay (FID). The FID is then turned into an NMR spectrum of the familiar type (such as Figure 16) by a Fourier-transform computer algorithm. The FTNMR, therefore, functions at the data analysis level much in the same manner as the previously described FTMS.

C. Structure Elucidation by NMR

As previously discussed, the fragmentation behavior of organic analytes observed in mass spectra can be interpreted from fundamental principles. However, for molecules other than the very simplest, such interpretations are seldom unambiguous. NMR spectra can also be interpreted from fundamental principles;[50,51] however, unlike mass spectra, every signal in an NMR spectrum can and should be interpreted. It should be possible, given sufficient NMR data, to unambiguously assign all signals in ^{1}H and ^{13}C spectra to individual H and C in an analyte molecule.

Such unambiguous assignments are not accomplished with the simple NMR experiment described above, or NMR spectra of the type shown in Figure 16. NMR is in fact not one but a family of related experiments as shown in Table 4, each providing different information about analyte molecular structure. For a typical structure elucidation, several NMR experiments are accomplished on an unknown analyte and the information interpreted as a whole. These NMR experiments are described in detail in various textbooks[50,51] and in a recent comprehensive review including pharmaceutical applications by Facchine and O'Connell.[52] An NMR structure elucidation on an unknown small molecule drug substance process impurity, for example, might include:

1. A ^{1}H-NMR spectrum (termed a one dimensional (1D)-^{1}H spectrum), providing information on the types and relative numbers of protons in the unknown.

TABLE 4 Summary of Nuclear Magnetic Resonance Spectroscopy (NMR) Experiments for Small Molecule Structure Elucidation (See References 6, 50)

General experiment type	Experiment acronym	Information available
^{1}H-NMR	• 1D-^{1}H	• Types and numbers of protons
^{13}C-NMR	• 1D-^{13}C	• Types of carbons
Modified 1D-^{13}C	• APT	• Protons attached to carbon (CH, CH_2, CH_3)
	• DEPT	
	• INEPT	
	• GATEDEC	
^{1}H–^{1}H correlation (2D-^{1}H)	• COSY	• H–H correlation, through bond
	• TOCSY	• Long-range H–H correlation, through bond
	• NOESY	• H–H correlation, through space
	• ROESY	• Long-range H–H correlation
^{1}H–^{13}C correlation (2D-^{13}C)	• HMQC	• Short-range H–C correlation
	• HMBC	• Long-range H–C correlation

2. A ^{13}C-NMR spectrum (termed a 1D-^{13}C spectrum), providing information on the types of carbons in the unknown (note that ^{13}C-NMR spectra are not quantitative due to relatively long relaxation times and the Nuclear Overhauser Effect[50]).
3. One or more ^{1}H–^{1}H correlation (or 2D-^{1}H) experiments (such as COSY) providing information as to which ^{1}Hs are attached to adjacent carbon atoms, or are near each other in space, in the unknown.
4. One or more ^{1}H–^{13}C correlation (or 2D-^{13}C) experiments (such as HMQC) providing information as to which protons are attached to which carbon atoms in the unknown.

The power of NMR to accomplish structure elucidation is unquestioned. The fact that NMR experiments are typically accomplished on samples dissolved in suitable solvents, and that the NMR spectrometer has no high vacuum, or other systems, that can be compromised by the solvent (in fact, the NMR sample never leaves the NMR tube during analysis and, therefore, never actually enters the spectrometer), make the combination of HPLC with NMR an attractive and potentially very powerful analytical tool. The questions to be addressed for this combination are:

1. Is NMR spectroscopy sensitive enough to acquire sufficient data from individual trace level analytes so as to allow for structure elucidation?
2. How does the use of HPLC as a sample inlet system for NMR affect the acquisition and quality of NMR data?
3. How does the use of the NMR spectrometer as a detector for HPLC affect chromatography method development?

The answers to these questions are critical, since analyte peak isolation/enrichment/purification is a viable and historically often applied alternative to LC/NMR.

D. LC/NMR: Structure Elucidation Tool or Laboratory Curiosity?

1. Background and Utility

The history and development of LC/NMR from the first published paper in 1978[53] to the year 2003 have been outlined and described by Elipe.[54] Advances in NMR spectrometer technology, including the availability of higher field superconducting magnets ($B_0 \geq 500$ MHz, proton resonance frequency), magnet shielding, and probe design, have made LC/NMR a viable commercial technology since about the middle 1990s. The issue of "sensitivity" is central to the effective application of LC/NMR. In this context, sensitivity is not defined as the ability of the NMR spectrometer to detect a signal from an eluting HPLC peak (as it might be defined for a UV detector, for example), but as the ability of the

NMR spectrometer to acquire sufficient data to allow for the unambiguous structure elucidation of a trace level analyte(s) in a complex mixture analyzed by HPLC.

Sensitivity in NMR depends on several factors, including the relative abundance of the magnetically active nucleus (e.g., ^{1}H at 99.985% and ^{13}C at 1.108%), the magnetogyric ratio (γ) of the magnetically active nucleus, the strength of the magnetic field (B_0), the number of analyte molecules contained within the exicitation/magnetization detection region (B_1) of the probe during data acquisition (termed the "active" region), and the background "noise" level during a given NMR experiment. With these factors in mind, consider the three available modes of operation for LC/NMR:[54] *On-flow*, *Stop-flow*, and *Loop-collection*. The *On-flow* experiment is analogous to an LC/MS experiment in that NMR spectra are acquired as eluting HPLC peaks flow through a specially designed "flow probe". Obviously, as the HPLC peak is of finite width, it is not possible to accomplish extensive signal summing during such experiments (which is the manner in which most "tube" NMR experiments are accomplished), giving this LC/NMR data acquisition mode the poorest "sensitivity". However, the *On-flow* experiment does allow for the acquisition of data, which can be displayed in a similar manner to LC/MS data. Feinberg[49] showed an in-line UV chromatogram from an LC/NMR analysis of a sample of ranitidine drug substance (1 mg on-column) spiked with three reference impurities at 10% (100 µg on-column), 1% (10 µg on-column), and 0.1% (1 µg on-column). His data also demonstrated that reasonable quality 1D ^{1}H-NMR spectra could be observed from the parent compound as well as the 10 and 1% spiked impurities in this *On-flow* experiment.

In the *Stop-flow* experiment, the HPLC flow is halted as an eluting peak enters the active region of the flow probe, following which various NMR experiments can be accomplished. In this acquisition mode, signal summing is possible, which results in greater "sensitivity". Figure 18 shows a 1D ^{1}H-NMR spectrum from the 1% spiked ranitidine impurity in Feinberg's LC/NMR experiments acquired in *Stop-flow* mode (note that Feinberg[49] also showed a 2D TOCSY spectrum from the 1% spiked impurity). Figure 19 shows a 1D ^{1}H-NMR spectrum from the 0.1% impurity, also acquired in *Stop-flow* mode. In *Loop-collection* mode, a special device is employed to collect each eluting peak from an HPLC analysis. Each peak can then be sent into the NMR spectrometer's flow probe for analysis. This acquisition mode also allows for signal summing during acquisition of NMR data resulting in increased "sensitivity" relative to *On-flow*. The differences in *Stop-flow* and *Loop-collection* relate to efficient use of spectrometer time and effects on chromatography.

This relatively simple spiked ranitidine example of an LC/NMR analysis suggests that the acquisition of 1D ^{1}H-NMR data is relatively

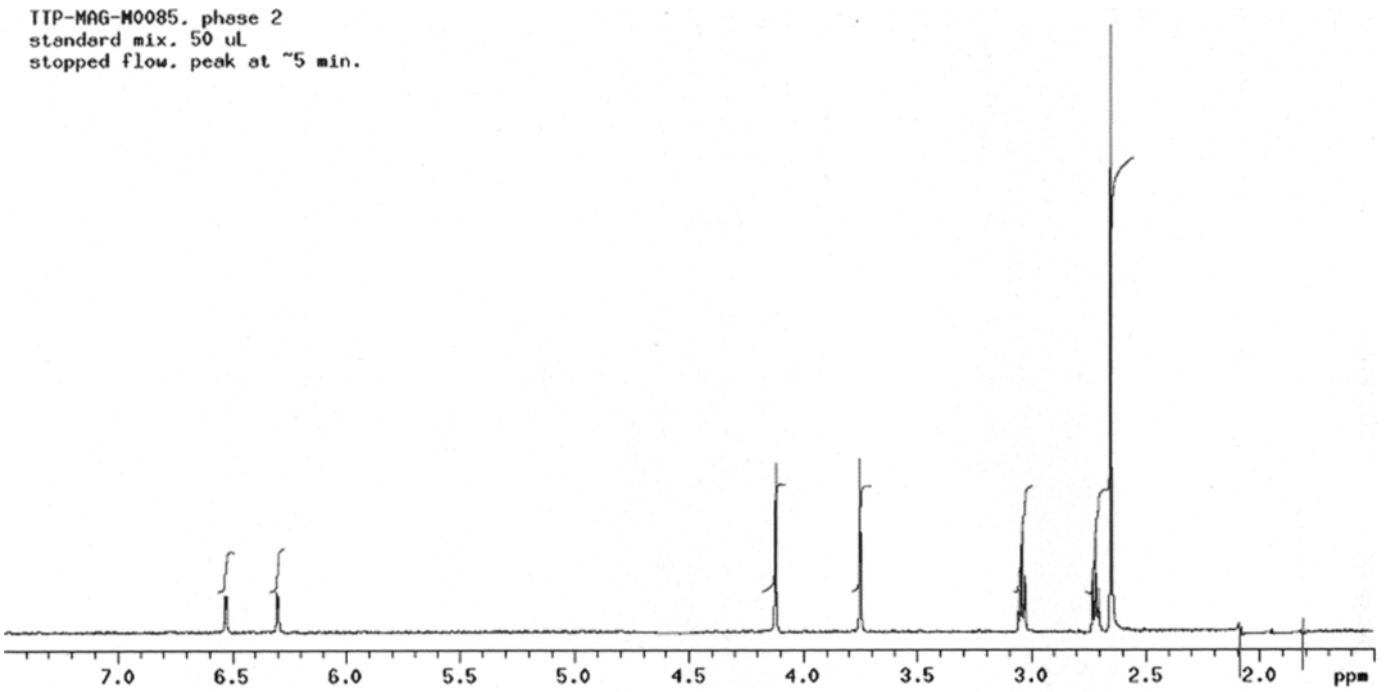


FIGURE 18 ^{1}H-NMR spectrum of the 1% spiked ranitidine-related impurity (*Stop-flow* LC/NMR).

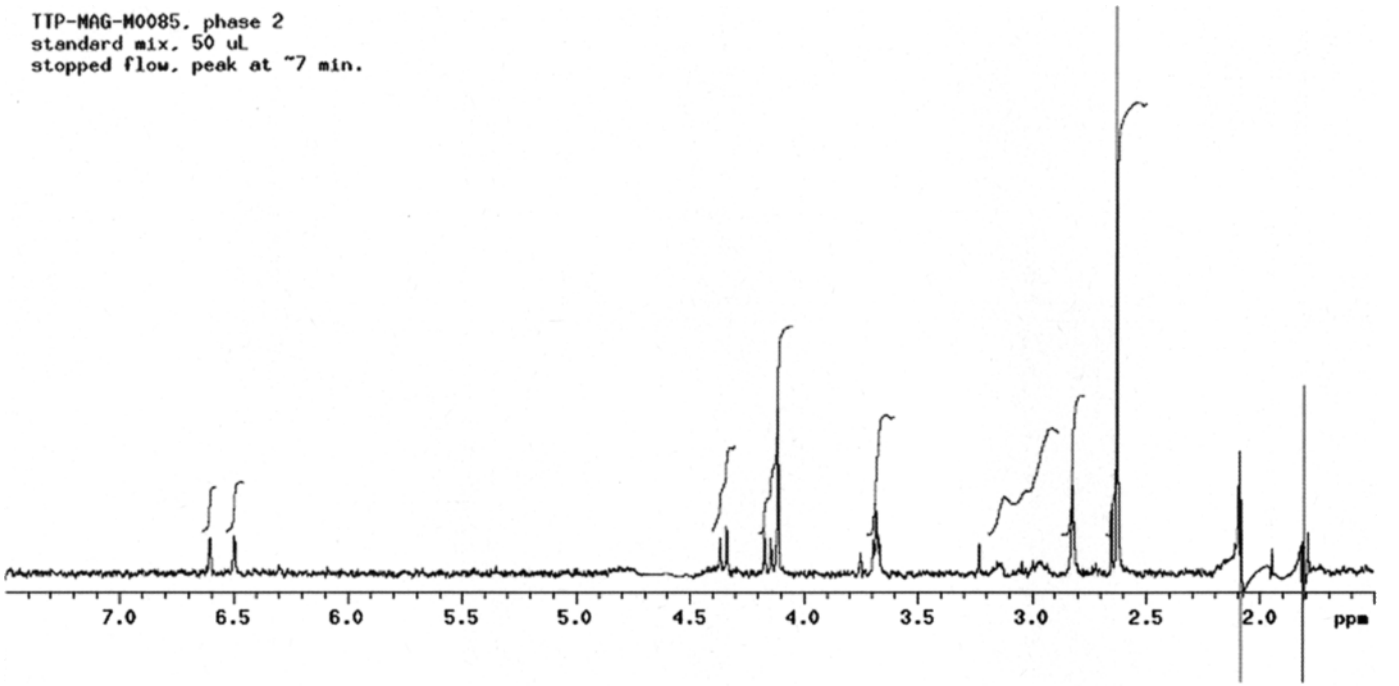


FIGURE 19 ^{1}H-NMR spectrum of the 0.1% spiked ranitidine-related impurity (*Stop-flow* LC/NMR).

routine for trace level analytes, with acquisition of 2D ^{1}H–^{1}H correlation data being possible at somewhat lower sensitivity. The literature suggests that a practical limit for the acquisition of 1D ^{1}H-NMR spectra from an LC/NMR experiment is between 0.5 and 1 μg for a typical small molecule analyte (molecular weight 300–500).[54,55] This estimate is supported by the ranitidine spiked impurities LC/NMR experiment described here and by Feinberg.[49] The acquisition of 1D ^{13}C-NMR spectra and 2D ^{1}H–^{13}C correlation spectra are generally considered not routine with LC/NMR for trace level analytes. The question then becomes: What structure elucidation is possible with ^{1}H-NMR spectra alone?

2. Chromatography and NMR: Cause and Effect

Thus far, we have said nothing about either the effects of employing HPLC on the NMR experiment and instrumentation, or the effects of NMR detection on HPLC method development. This is with good reason, since without sufficient sensitivity to solve real problems the answers to these two questions are academic. Having established that sensitivity is adequate to address at least some real problems, we can now address unique issues related to the combination of HPLC and NMR. These are as follows:[54,55]

1. *The use of protonated solvents for reverse-phase HPLC mobile phases is not possible with LC/NMR.* If protonated reverse-phase solvents (e.g., water, acetonitrile, methanol, etc.) were employed for LC/NMR, their resonance signals would dominate any ^{1}H-NMR spectra acquired and swamp the relatively weak signals from trace level analytes. The solution to this problem is to use deuterated solvents (e.g., D_2O, CD_3OD, CD_3CN, etc.) for LC/NMR experiments. Deuterated solvents can be expensive and can potentially compromise chromatographic separations with peak broadening and retention time shifts relative to protonated solvents. Further, H/D exchange reactions will result in loss of NMR spectral information on exchangeable (or active) protons in analyte molecules. This information would be available for structure elucidation purposes were the analyte to be analyzed in $CDCl_3$ or some other similar NMR solvent. Ionic buffers in the mobile phase have the potential to detune the probe, resulting in spectral line broadening.
2. *Even with deuterated mobile phases, solvent suppression techniques are required.* The best available deuterated solvents still contain relatively significant levels of the corresponding protonated species; and it is, therefore, necessary to employ so-called "solvent suppression" techniques to remove the corresponding signals from trace level analyte ^{1}H-NMR spectra. Various instrumental techniques, including the WET solvent suppression technique,[54] are commercially available. Unfortunately, solvent

suppression also results in the suppression of analyte proton signals near the suppressed solvent lines with an accompanying loss of spectral information about the analyte. Further, phase artifacts (negative lines) are possible as well as effects on the integration of proton signals resulting in lack of precision in proton counting.

3. *HPLC mobile-phase gradients can cause problems with magnetic field homogeneity.* Solvent mixing in the NMR flow probe can cause problems with "shimming" the magnet, with a resulting loss of optimum performance of the spectrometer. The literature suggests that mobile-phase gradients of greater than 2–3% per min are not advised for LC/NMR.[54] Further, ^{1}H-NMR spectra of analyte molecules can be a function of the solvent in which the analyte is dissolved, which means that an analyte eluting under one set of mobile-phase conditions may have a ^{1}H-NMR spectrum, which appears different than when it elutes under a different set of conditions. In the case of process impurity structure elucidation, it is often useful to compare the spectra of trace level impurities with that of the parent in the same LC/NMR analysis. In a gradient HPLC analysis, each impurity and the parent compound would elute in a different solvent system with potential effects on ^{1}H-NMR spectral appearance.

An interesting solution to the problem of mobile-phase gradients has been proposed by Jayawickrama et al.[56] Their technique involves using a second equal but reverse mobile-phase gradient which is combined with the analytical gradient before eluting peaks reach the NMR flow probe. In this way, the solvent composition reaching the active region of the NMR probe is kept constant (i.e., isocratic). These workers were able to show significant improvements in proton chemical shift stability and spectral line width stability using the compensating gradient technique. However, they also noted an overall loss of signal-to-noise (i.e., sensitivity), which they attributed to dilution by the compensating gradient. There is clearly a trade-off to be had between sensitivity and spectral stability when using mobile-phase gradients with LC/NMR.

4. *Many (if not most) LC/NMR experiments require signal summing in order to achieve adequate signal-to-noise and, therefore, sensitivity.* This presents an obvious problem for *On-flow* LC/NMR analyses. For *Stop-flow* and *Loop-collection* LC/NMR experiments, the spectrometer time required to collect adequate data can be significant. Since signal-to-noise increases as $\sqrt{n}$, where n is the number of rf pulse excitations, it is possible to require up to 24 h to accomplish a single experiment on a single analyte peak (either with *Stop-flow* or *Loop-collection*). The effect on productivity for an NMR laboratory with only a few spectrometers is clear. The *Stop-flow* mode also has the potential

to cause chromatography peak broadening as diffusion takes place on the HPLC column during *Stop-flow* data acquisitions. Peak broadening effects can reduce the amount of an analyte peak, which gets into the active volume of the flow probe with resulting sensitivity issues.

5. *The physical proximity of the HPLC system with the NMR superconducting magnet can be an issue.* Since the HPLC contains metal and moving metal parts, difficulties will be encountered if it is too close to the superconducting magnet. In the days of unshielded magnets, this required the HPLC to be at least 1.5–2 m away.[54] With modern shielded magnets, 30–50 cm is adequate;[54] however, this is still a long way for a detector to be from the end of the HPLC column.
6. *In order to ensure that an adequate amount of analyte is in the active region of the NMR flow probe, it is often necessary to "overload" the HPLC system with sample.* Overloading can cause poor chromatographic resolution, retention time shifts, peak splitting, and other deleterious effects. There needs to be a compromise in any given situation between sample loading and chromatographic effects for an adequate LC/NMR analysis. The chromatographer must find a way to get the maximum amount of analyte in the most narrow peak possible.

From the above discussion, it is clear that effective use of LC/NMR is a constant battle for sensitivity.

3. LC/NMR Pharmaceutical Applications and Cost–Benefit Analysis

In spite of its many issues and unique "features", which impact both HPLC method development and the use of NMR as a detector for HPLC, LC/NMR has been used to address many problems in both drug discovery and pharmaceutical development. The interested reader is directed to the previously cited articles by Facchine and O'Connell,[52] as well as the comprehensive reviews by Hicks,[57] Keifer,[58] Stockman,[59] and Corcoran and Spraul.[60] Noteworthy applications include: the identification of metabolites from a *ras* farnesyl transferase inhibitor in rats and dogs using *Stop-flow* LC/NMR by Elipe;[54] and the identification of a pseudo-dimeric bulk drug impurity using *Stop-flow* LC/NMR with 1D ^{1}H-NMR and 2D TOCSY spectra by Potts et al.[61] It is clear from the currently available literature (including the discussion of pharmaceutical impurity identification by Cummings et al.[62]) that LC/NMR is best applied to trace level analyte structure elucidation problems, which can be solved with 1D and 2D ^{1}H-NMR experiments (i.e., ^{13}C-NMR is not required). This requires an analyst to be able to predict with a reasonable degree of scientific certainty which problems fall into this category.

Sharman and Jones[55] have published a critical investigation of the use of LC/NMR for pharmaceutical impurity investigations in which they conclude, based on their own laboratory studies and experiences as well as a study of the scientific literature, that LC/NMR is not always the most efficient approach for structure elucidation of trace level impurities. They contend that so-called "classical" approaches involving chromatographic peak isolation and purification, followed by tube NMR experiments are often more cost-effective. Even Elipe,[54] in her metabolite identification study, noted that in the case of one particular metabolite it was necessary to isolate the peak for 2D ^{1}H–^{13}C correlation experiments to elucidate the structure. Based on the currently available scientific literature and our own experiences, it appears that LC/NMR is best applied to structure elucidation problems where the number of possible structures is limited to only a few (perhaps <5), and/or the structural modifications to the parent molecule are relatively minor. Structural possibilities can be narrowed by a complete understanding of the sample history, and a complete application of LC/MS to the problem.[62] If the number of structural possibilities remains excessive, then it is recommended that peak isolation and purification be accomplished for a complete NMR structure elucidation (i.e., NMR proof-of-structure).

It is important to emphasize that advances in technology will in all probability increase the applicability of LC/NMR for trace level structure elucidation. Technology advances include the development of higher sensitivity flow probes, including microflow probes as discussed by Olson et al.[63] and cryoflow probes to reduce noise as discussed by Spraul et al.,[64] and the use of improved data acquisition techniques (see Shapira et al.,[65] for example). It is also possible to use high sensitivity flow probes for flow injection analysis of isolated HPLC peaks, which may allow complete NMR structure elucidation to be accomplished on relatively small isolated/purified sample amounts (i.e., $<1\ \mu g$).

4. LC/MS/NMR

One final hybrid instrument, which has seen pharmaceutical application, is the combination of HPLC with both MS and NMR in the so-called LC/MS/NMR (or LC/NMR/MS, depending on your point of view). Typically in this combination, the eluent from the HPLC column is split with a relatively small amount going to a mass spectrometer operating in ESI mode, and the rest going to the NMR spectrometer (remember that the MS has enormous sensitivity compared with the NMR). The acquisition of both data types allows for an unambiguous correlation of NMR spectra with a particular trace level analyte under investigation, as confirmed by the mass spectrometer. However, one must remember that deuterated mobile phases must still be employed, resulting in H/D exchange, which will be reflected in the resulting mass spectra. Details of

LC/MS/NMR are discussed by Elipe,[54] who accomplished both *On-flow* and *Stop-flow* experiments on a series of flavonoids. At this time, the majority of LC/MS/NMR applications are in drug discovery, including Combinatorial Chemistry and high-throughput screening, and drug metabolism/pharmacokinetics and metabolite identification. Such applications often involve flow-NMR rather than true LC/NMR. For additional discussion, the interested reader is referred to the review by Corcoran and Spraul.[60]

As with LC/NMR, one must consider the relative costs and benefits of LC/MS/NMR for a particular pharmaceutical application. The cost of the instrument itself, not to mention the expert personnel and infrastructure required to support it, is significant. In high-throughput flow-NMR applications for drug discovery as well as for metabolite identification, the utility is clear. However, for trace level impurity identification, the equation is more difficult to balance. In the time it takes to accomplish one LC/MS/NMR experiment, many LC/MS experiments could be accomplished, and one could perhaps be well on the way to isolating and purifying individual impurity peaks. As with LC/NMR application, the choice requires expert evaluation of each individual problem.

IV. CONCLUSIONS

The hyphenated techniques LC/MS and LC/NMR have greatly advanced, and continue to advance, the application of HPLC to the pharmaceutical sciences. There is not a single pharmaceutical application area in which LC/MS has not had an impact, and the potential and promise of LC/NMR are significant. For problems in qualitative analysis, both LC/MS and LC/NMR are contained in systematic problem-solving strategies such as that proposed by Norwood and Qiu.[48] Each of these techniques has its own individual method development issues and constraints, and it is up to chromatographers to understand and consider these in order to fully exploit the significant capabilities of hyphenated techniques.

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8
HPLC SAMPLE PREPARATION

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ABSTRACT

Sample preparation is a critical step in the analysis of pharmaceutical products. The samples being prepared can come in many forms, as neat chemical compounds such as active pharmaceutical ingredients (API) or in complex matrices that support discovery and toxicological

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studies requiring the isolation of analytes from reaction byproducts, animal feed, biological fluids and tissues, waste streams, or environmental samples. All of these require sample preparation prior to analysis, which is the point in the method where the analyst will manipulate samples and have a direct effect on quantitative analysis. Since modern instrumentation is designed in many ways to self-check to ensure that it is working properly, sample preparation becomes a major source of laboratory deviations. Since sample preparation errors are often subtle and not easily diagnosed the proper application of these techniques is critical.

All sample preparation methods have their theoretical basis in chemical equilibrium, so an understanding of the fundamentals of chemical equilibrium is critical for developing, troubleshooting and investigating pharmaceutical methods is essential for the analyst. This chapter provides an overview of the fundamentals of extraction and chemical equilibrium, the traditional sample preparation techniques commonly employed in the pharmaceutical laboratory such as, direct injection, liquid liquid extraction and solid phase extraction along with some of the additional sample preparation methods that are establishing their place in lab, including solid phase microextraction, accelerated solvent extraction and microwave extraction, or are used to solve unique sample preparation challenges as in derivatization.

I. INTRODUCTION

Sample preparation is a critical step in the analysis of pharmaceutical products. The samples being prepared for high performance liquid chromatography (HPLC) analysis of pharmaceuticals can come in many forms, as neat chemical compounds such as active pharmaceutical ingredients (API) or drug substances, in a simple tablet or liquid matrix (the drug product) comprising a few solid or liquid excipients, complex drug delivery systems and possibly pharmaceutical devices. In addition to developing methods to support these specific dosage forms, methods are required to support discovery and toxicological studies requiring the isolation of pharmaceutical analytes from reaction byproducts, animal feed, biological fluids and tissues, waste streams and environmental samples. All of these require sample preparation prior to analysis. Sample preparation can be as simple as the transfer of the analytes from the source container to an HPLC sample vial or complex requiring multiple steps such as extraction, concentration, cleanup, solvent transfer and chemical modification. Regardless of the complexity, when the final sample is ready for injection into the HPLC it should be:

- an accurate representation of the original sample;
- in a solvent that is the same as the mobile phase or at least compatible with the mobile phase;
- in a solvent that provides sufficient stability for the duration of the analysis and any potential investigations;
- free of particulates that could damage the HPLC system;
- at a concentration appropriate for the method of detection; and
- in a chemical form that is compatible with the mode of detection.

Although liquid chromatography (LC) is a mature technique with commercially available instrumentation and automated injection, the sample preparation methods are often labor intensive and require a trained

analyst.[1] Further, the use of proper analytical techniques, equipment, measurement principles, analyte concentrations[2] and documentation are necessary for the generation of accurate reliable results. The use of improper weighing techniques, buffer and mobile phase preparations, transfer methodology, chemical reagents and glassware will lead to the introduction of impurities, loss of sample and decrease in precision, respectively.[3] The lack of a proper documentation will often result in difficulties when investigations occur or during the transfer of the method from the development lab to the end user or quality control lab.

Finally, it is important to remember that sample preparation is an integral part of method development and validation. In order to develop a rugged method, a proper statistically valid sampling plan should be developed[4] and each step of the sample preparation process should be validated in accordance with validation guidelines.[5–7]

II. FUNDAMENTALS OF EXTRACTION AND CHEMICAL EQUILIBRIUM

A full understanding of the myriad pharmaceutical sample preparation techniques requires knowledge of chemical equilibrium theory, which underpins nearly all sample preparation and handling methods and techniques. Reproducible chemical analysis generally requires that the system being studied is at equilibrium or is driving toward equilibrium. The bulk of investigations that result from laboratory sources other than obvious mishaps or instrument failures result from the analytical system not being brought fully to equilibrium, for example, not completely dissolved or not brought to the correct temperature. In this section, the fundamentals of chemical equilibrium will be discussed, along with examples of the consequences of not considering equilibrium in sample preparation and analytical studies. Liquid–liquid extraction (LLE), the most classical sample preparation technique, with which nearly all analysts are very familiar, will be used to illustrate the key points inherent in all sample preparation methods. The key fundamentals of kinetics and equilibrium are discussed in most undergraduate general and physical chemistry textbooks. Both of these should be a regularly used part of every analyst's and investigator's bookshelf.

A. Fundamentals of Chemical Equilibrium

All students of chemistry become familiar with the basic concept of chemical equilibrium early in their studies. In the case of extraction, the simple chemical equation shown below describes the transfer of a single analyte between two immiscible liquid phases:

$$A(\text{phase 1}) \rightleftharpoons A(\text{phase 2}) \quad (1)$$

where *A* represents the analyte and phase 1 and phase 2 represent the two liquid phases. The double arrows indicate that analyte molecules are continuously transferred in both directions between the two phases at all times. When the rates of the forward and reverse processes are equal, there is no net change in analyte mass and concentration in both phases and the system is said to be at equilibrium. Since significant time might be required for this condition to be achieved, there are kinetic factors that affect extraction and all sample preparation methods. Factors that may affect the kinetics of coming to equilibrium in sample preparation include:

- Temperature
- Shaking or agitation rate and process
- Volume of the two phases
- Diffusion coefficients of the analytes within the two phases

The mass of analyte that can be transferred from one phase into another is given by the concentration equilibrium constant of the above chemical equation:

$$\tilde{K}_c = \frac{[A_2]}{[A_1]} \tag{2}$$

where the values in brackets represent the concentration of the analyte in each phase and by the thermodynamic equilibrium constant, which is more rigorous and includes the activity of each component.

$$K^o = \frac{[A]_2 \gamma_2}{[A]_1 \gamma_1} \tag{3}$$

In dilute solutions, usually 10 mM or less, the activity coefficients are equal to 1 and the equation reduces to the concentration equilibrium expression. However, in all situations, differences in chemical activity account for variation in results when analytical samples are prepared under different conditions such as solvents, pH or in the presence of interferences not found in the standards. This is common when the samples are in complex matrices such as biological fluids, devices or complex products.

Although equilibrium expressions are written to imply that the equilibrium constant is a function of concentrations, in fact, the opposite is true: the concentrations obtained and therefore, the mass of analyte transferred in any sample preparation method is a function of the value of the equilibrium constant. Fundamentally, the equilibrium constant is a function of the thermodynamics of the system being studied, as shown below:

$$K_c = e^{-\Delta G^o/RT} \tag{4}$$

Equation (4) relates a concentration equilibrium constant to the Gibbs' free energy and temperature of the system. It is clear that factors that affect thermodynamics and activity are also important in sample preparation. In pharmaceutical analysis, these may include:

- Temperature
- Choice of extraction solvent(s) and media
- pH
- Ionic strength
- Analyte concentration
- Presence of interferences or impurities
- Competing chemical reactions

Finally, the consequences of changing a system that has been brought to equilibrium must be considered. Changes may be as simple as a change in the ambient temperature or the removal of analyte from one phase of the extraction, or they may be as subtle as the slow degradation of the samples over time. This is governed by the well-known Le Chatelier's Principle, which states that if a system at equilibrium is somehow changed, the concentrations of reactants and products will shift to restore equilibrium.

B. Solubility, pH, and Solution Preparation

Dissolving and preparing solutions is perhaps the most common set of techniques performed by pharmaceutical analysts. Pharmaceutical samples, such as raw materials and finished products are generally much more concentrated than needed or appropriate for instrumental analysis, so they must be diluted and/or extracted in sample preparation. Although generally simple, these procedures generate a very large portion of the out-of-specification (OOS) or out-of-target (OOT) results leading to investigations. Nearly all pharmaceutical analysis methods require the preparation of solutions either of the analyte(s) themselves or as diluents or mobile phases. There are several equilibrium-based processes that may affect solution preparation, which include

- Solubility and extraction
- pH in buffer preparation and solubility
- Matrix and interference effects
- Proper handling and use of glassware

C. Solubility and Extraction

Solubility considerations pervade pharmaceutical analysis, although the bioavailability properties that make a compound an excellent drug candidate may limit choices for appropriate analytical solvents. For

example, low-polarity lipid-soluble compounds are often difficult to dissolve in the polar mobile phases and diluents required for reversed-phase HPLC. Thus, it is important to be aware of all available solubility data for all analytes and as many interferences as possible in a sample.

For molecular analytes, such as most drugs, the solubility equilibrium may be expressed by the simple expression shown in Equation (1), with the left side representing the pure solid or liquid, with a concentration of 1 by convention and the right side representing the solution phase. Thus, when the equilibrium expression is calculated, the solubility is expressed as a concentration. Analysts should be aware that different solubility units such as g/100 mL or g/L may be used within the same method. Solubility data can be used to estimate both the recovery from a proposed extraction or dissolving scheme or the effectiveness of separation from extraction or dissolving.

For simple dissolution of a single component, the mass that can be dissolved in a given sample preparation scheme is given clearly by the solubility, which can be simply corrected for the amount of solvent actually available. However, the actual solubility may differ from literature data due to differences in experimental conditions between the new method and the solubility data. Also useful is the use of solubility data to determine the ability to use extraction or dissolving to separate sample components. In principle, compounds can be separated effectively if one component is highly soluble in the extraction solvent, while the other is not.

For example, in an LLE, if two compounds are dissolved in water and separation is desired by extracting into an organic solvent, solubility can be used to estimate the effectiveness of the proposed scheme. If the extraction is from water to hexane and

- Compound A has solubility 10 g/100 mL in water and 1 g/100 mL in hexane
- Compound B has solubility 2 g/100 mL in water and 10 g/100 mL in hexane

and if 1 g of each compound is dissolved in 100 mL water and shaken with 100 mL of hexane, the results presented in Table 1 are obtained. Interestingly, as a result of equilibrium theory, if the same volume of hexane is used, but the extraction is performed in four separate 25-mL steps, with

TABLE 1 Calculated Extraction Results for the Single LLE of Two Compounds A and B with Different Solubilities

Compound	K	Mass extracted into hexane (g)	Mass remaining in water (g)
A	0.1	0.09	0.91
B	5	0.83	0.17

TABLE 2 Calculated Extraction Results for Multiple LLE of Two Compounds A and B with Different Solubilities

Compound	Mass extracted into hexane (g)	Mass remaining in water (g)
A	0.09	0.91
B	0.96	0.04

TABLE 3 Calculated Extraction Results if the Combined Extract Shown in Table 1 is Washed, or Back-Extracted with 50 mL of Water

Compound	Mass into hexane (g)
A	0.02
B	0.87

the entire 100 mL of water extracted with 25-mL hexane and the resulting extracts combined, the results presented in Table 2 are obtained.

This is a simple example of the very high extraction efficiency that can be obtained from multiple extraction and is the reason why continuous extraction methods, such as steam distillation or Soxhlet extraction, are commonly used in environmental analysis and also provides the basis of the very high separating power achievable in chromatography, which can be viewed as thousands of individual extractions, in sequence.

Finally, in both dissolving, extraction and filtering, "wash" steps are often used to remove much of the remaining traces of the unwanted component, in this case compound A, seen in the second line of the above table. If the combined extract shown above is washed, or back-extracted with 50 mL of water, the results presented in Table 3 are obtained.

A negative consequence of equilibrium theory is also seen in the above example. Note that as each sample preparation step is performed, the total amount of the analyte extracted (in this case, compound B) decreases. This occurs in any method in which equilibrium theory applies and additional losses result from glassware holdup and non-quantitative sample transfers.

D. pH in Buffer Preparation and Solubility

In pharmaceutical sample preparation, pH adjustment is possibly the most commonly used tool for adjusting and controlling the solubility of analytes and interferences. For ionizable compounds, the pH of the solvent system will have profound effects on the solubility, the chemical form of the compound and its behavior in both sample preparation and instrumental analysis, especially in LC. The acid–base equilibria that govern the pH of pharmaceutical samples are the same as those discussed

in most undergraduate general chemistry and quantitative analysis textbooks, so they are not repeated in detail here. In short, control and equilibrium of the ionization of the weak acids and bases that are common pharmaceutical analytes is a critical component of effective sample preparation. Typically, analytes are either fully ionized by adjusting solution conditions to high pH for acids or low pH for bases, or ionization is fully suppressed by adjusting to low pH for acids or high pH for bases.

In developing a buffer system, the need to either fully ionize or fully suppress ionization of the analyte governs preparation of the buffer. For most pharmaceutical applications, buffer preparation is governed by the Henderson–Hasselbalch equation, which relates the desired buffer pH to the amount of an acid (acid) and its corresponding salt (base) that are mixed to make the solution:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{base}]}{[\mathrm{acid}]}$$

Buffers have the capacity to resist changes in pH due to additions to the solution such as analytes; this capacity is related to the buffer's total ionic strength, which for pharmaceutical applications usually need not be greater than 10 mM. The Henderson–Hasselbalch equation is effective for determining buffer components within about 2 pH units of the buffer $\mathrm{p}K_a$. If this equation is rearranged to solve for the base–acid ratio, it can be used to determine the percent ionization of any compound in solution for which the solution pH and analyte $\mathrm{p}K_a$ are known. As a result, for an acidic analyte to be termed fully ionized, the pH of the solution must be at least two pH units above the $\mathrm{p}K_a$ of the analyte, or for ionization to be fully suppressed, the pH should be at least two pH units below the analyte $\mathrm{p}K_a$. This idea is often used in HPLC method development and is the key reason why HPLC methods for acidic analytes are usually carried out at low pH.

E. Matrix and Interference Effects

Nearly all pharmaceutical samples include a range of matrix interferences that may include a variety of materials or excipients, depending on the dosage form. Tablets and powder-filled capsules generally have the fewest interferences, while creams, controlled release formulations and drug-coated devices may involve very complex matrices, including the device itself. Formulations that provide the desired bioavailability, selectivity and marketability may provide the largest hindrance in developing sample preparation methods. For example, drugs that are designed to be time-released may be encapsulated into a matrix that provides slow release under biological conditions. This encapsulation, designed to slow this dissolving process biologically, will also slow it analytically. A classical example is found in the assay of simple aspirin tablets, which was classically performed by acid–base titration. If the titration was performed or stopped prior to complete dissolving of the aspirin tablet, a low analytical assay result is obtained.

F. Proper Handling and Use of Glassware

Finally, in any discussion of the equilibrium and solution chemistry aspects of sample preparation, comments about the fundamental use and handling of glassware and samples is required, as these affect both quantitative analysis and analyst safety. Prior to performing any analytical method or experiment, the analyst must become familiar with all safety and personal protection considerations relating to the method. Also, all glassware to be used should be checked and any that appears damaged should be discarded and replaced. The United States Pharmacopeia (USP) requires that all assays be performed using Class-A volumetric glassware; it is recommended that, even if some procedures do not require Class-A glassware, all volumetric glassware in the laboratory be Class-A, to avoid any mistakes and confusion. Class-A glassware has tolerances for volume accuracy that are traced to calibration procedures developed by the National Institutes of Standards and Technology.

Volumetric glassware should be handled with great care. A 100-mL volumetric flask is a five-significant digit apparatus, making it one of the most precise devices in the laboratory; it should be treated as such. Incorrect practices with volumetric glassware include:

- Cleaning with harsh detergents or strong acids or bases that etch glass
- Use of a hot plate to assist in dissolving
- Drying the flask with acetone or gas flow
- Drying the flask with a paper towel
- Mis-matching of flasks and lids causing leaks
- Cross-contamination by placing the lid on the bench top or pipetting into the wrong flask; only one flask should be open at a time
- Incorrect handling of blow-out or non–blow-out pipettes; know which one is being used
- Improper reading of the meniscus; read the bottom of the meniscus and be sure the device is on a flat surface
- Use of dirty glassware; the rinse solvent should cleanly run out of the flask or pipette
- Improper rinsing; volumetric glassware is best rinsed with the sample solvent or diluent

III. CHOICE OF SAMPLES PREPARATION

In general, sample preparation techniques have been developed or designed to handle samples by the form they are in gas, liquid or solid. Gas samples are analyzed by gas chromatography (GC) and will not be discussed. For neat liquid and solid samples the analytes may be dissolved directly into an appropriate solvent at the correct concentration for the type of analysis to be performed, filtered and analyzed. For analytes in a liquid sample matrix such as syrups or saline solutions the analyte is typically in an aqueous matrix and the sample is either analyzed directly or

TABLE 4 Sample Preparation Approaches Employed in the Pharmaceutical Industry

Liquid	Solid
Direct injection	Dissolving followed by liquid technique
Particulate removal Filtering or centrifuging	Grinding followed by dissolving followed by a liquid technique
Liquid–liquid extraction	Soxhlet extraction
Solid-phase extraction	Accelerated solvent extraction
Solid-phase micro-extraction (SPME)	Microwave-assisted extraction
Derivatization	Supercritical fluid extraction

extracted from the liquid by partitioning into an organic phase, which can be liquid or solid. For solids such as, tablets, capsules, feeds and natural products, the analytes must be removed from the solid matrix and dissolved into a liquid compatible for analysis. The common techniques for extraction of analytes from solid matrixes are listed in Table 4 and all have a similar approach – dissolve or suspend the matrix in a solvent, separate that solvent from the insoluble materials and then analyze the isolated solution or analyte. Regardless of the type of sample to be analyzed and the technique used, the goals of sample preparation are to cleanup or concentrate the sample prior to analysis. Each of the techniques listed in Table 4 will be described to provide the basic concepts of their use.

IV. DIRECT INJECTION

The simplest form of sample preparation is direct injection of the analyte into the HPLC system. Even if this is possible there will be sample preparations required unless the analyst can sample directly from the sample container, which is rare. Normally, the sample must be transferred to a sample vial compatible with the HPLC injection system. Even this simple step is considered sample preparation. While this is straightforward the choice of vial used for analysis may have an impact on the method if the analytes are not stable or if they interact with the container. The analyst should demonstrate through validation that there is no loss of sample due to analyte instability or interactions with the container. Furthermore, although samples may appear to be completely dissolved it is a good practice to centrifuge or filter the samples to remove particulates that may damage the LC injectors or plug the column inlet.[8] A large number of filters are commercially available and the analyst should check the vendor specifications to ensure compatibility of the filter with the solvents being

used. Additionally, the analysis of blank extractions and performing recovery studies to demonstrate that the filters do not affect the assay results is an important step in method development and validation. Filters can introduce impurities and adsorb the analyte of interest.

V. LIQUID–LIQUID EXTRACTION

LLE is a classic sample preparation technique, and as described in Section II is the basis for most modern extraction methods. LLE involves the transfer of dissolved analytes from one liquid phase to another; typically LLE involves the transfer of analytes from an aqueous phase to an organic phase for purposes of pre-concentration or cleanup, or to move the analyte to a solvent compatible with the method of analysis. The main requirement is that the two liquid phases be immiscible. In general, analytes with limited solubility in an aqueous media are transferred from an aqueous phase to an organic solvent by placing both solvents in a flask or separation funnel, shaking and then removing the organic layer for analysis. While the basic technique is straightforward, understanding the fundamentals of chemical equilibrium is essential when troubleshooting extraction methods where the recoveries or precision are not meeting the expected validation criteria of the method.

LLE has long been associated with the use of large volumes of solvents (hundreds to thousands of milliliters) and long extraction times. Modern sample preparation techniques have been developed to address this and is an attempt to replace LLE. However, due to the effectiveness of LLE, rather than employ alternative sample preparation methods such as solid-phase extraction (SPE) or solid-phase micro-extraction (SPME), many analysts have chosen to miniaturize the technique[9] performing extractions using only a few hundred micro-liters of sample and extraction solvent. One effective approach, semi-automated LLE for biological samples, uses a 96-well LLE as shown in Figure 1. Here, up to 96 micro-LLE can be performed at once by using an automated system to introduce the internal standard, make pH adjustments and add organic solvents. Following shaking, the organic layer can be removed and transferred to a clean 96-well plate where the samples are dried and reconstituted for analysis by LC/MS.[10] One example is the analysis of plasma samples where LLE is performed in 1.2-mL, 96-well format micro-tubes.[11] Other micro-systems have been developed to perform micro-size LLE where the contact between the aqueous phase and the organic phase, and the separation of the phases is carried out in a micro-batch glass extraction chamber. The sample, any reagents and organic solvent are introduced into a glass extraction chamber using a peristaltic pump where air carries the solutions to the chamber. The system allows for a 2–10-fold enrichment factor.[12] Another interesting

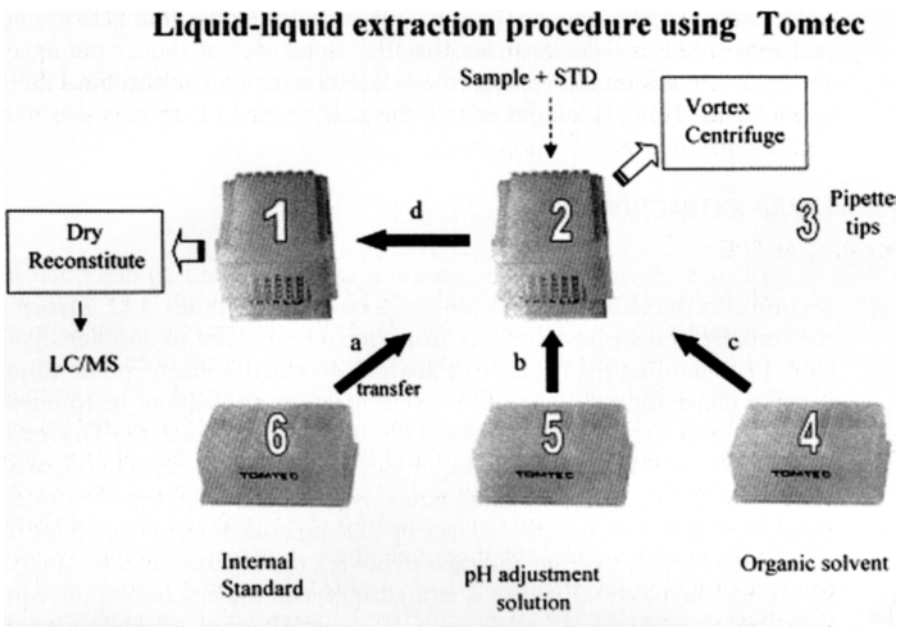

FIGURE 1 **Conceptualized semi-automated 96-well LLE procedure using Tomtec Quadra-96 liquid handling workstation. Refer to text for a description of the operation.[10] (Reproduced with permission from Elsevier.)**

approach is hollow fiber liquid-phase micro-extraction (LPME). This method submerges an Accurel Q3/2 polypropylene tubular membrane (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size), which is filled with acceptor solution, into the sample. After the analytes pass through the walls of the fiber into the extractor solution, the solution is transferred to an HPLC sample vial for analysis.[13] For simpler automation micro-LLE (less than 1 mL of extraction solvent) the analyst can simply use a micro-volumetric flask, conical test tube or an HPLC sample vial.

VI. SOLID-PHASE EXTRACTION

SPE is a separation technique that uses a small cartridge packed with a sorbent (stationary phase) to trap the analytes of interest from a liquid matrix. Once trapped on the sorbent, the analytes are then eluted using a strong solvent. The mechanism is essentially an HPLC separation using mobile phases at extreme capacities. There are numerous publications that describe the application of SPE in pharmaceutical analysis for sample pre-concentration,[14,15] cleanup of mobile phases,[16] isolating analytes from biological samples like urine,[17] human plasma[18] and blood,[19] or to

transfer the analyte to a solvent compatible with the type of analysis to be preformed. SPE is often compared to LLE since both of these techniques are used to transfer analytes from a weak to a strong solvent. Since SPE is similar to HPLC, it has potential advantages over LLE such as selective extractions and ease of automation.

A. Principles of SPE

SPE follows the same principles as LC to extract analytes from an aqueous sample onto a solid sorbent. Figure 2 illustrates this basic principle of SPE, regardless of the chosen retention mechanism – normal phase, reversed phase or ion exchange. Analytes are sorbed under conditions of low solvent strength and high retention and then eluted under conditions of high solvent strength and low retention.[20] For pharmaceutical samples the analytes are typically in an aqueous matrix (weak solvent) so a typical extraction method would use a reversed phase sorbent to trap the analytes and a small volume of an organic solvent ideally acetonitrile or methanol (strong solvent) to elute them from the sorbent. The distribution constant (K) of the analyte between solid sorbent and the aqueous matrix determines the amount of analyte extracted. For strongly hydrophobic compounds where the partition ratio is $>10^3$, nearly 100% of the analyte will be adsorbed from the sample. For semi-polar compounds, the analyte recovery will be lower due to their higher affinity for the aqueous sample matrix.[20] Low analyte recoveries can be compensated for using the techniques discussed below or by applying the principles presented earlier in Section II.

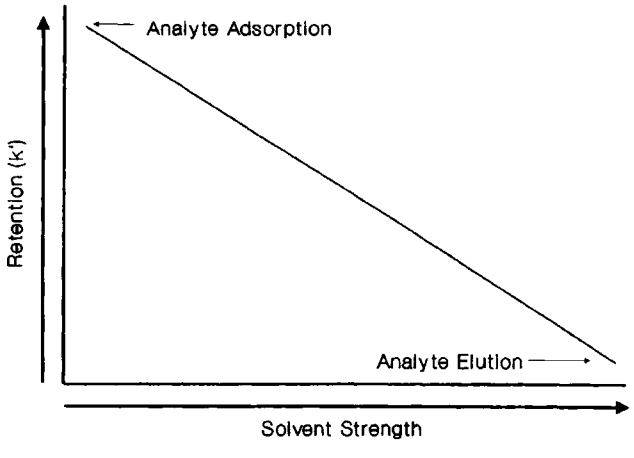

FIGURE 2 Graph of retention versus elution strength for a solid-phase extraction sorbent.[20] (Reproduced with permission from John Wiley & Sons, Inc.)

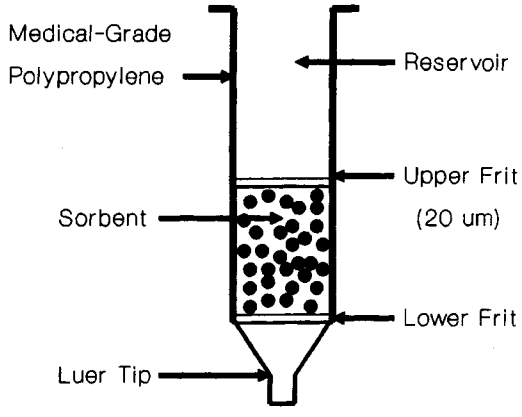

FIGURE 3 **Schematic of a solid-phase extraction cartridge. Typical dimensions for a 3-mL cartridge are 5.5 cm × 1 cm. Samples are poured into the top of the cartridge and are moved through the sorbent by vacuum or pressure.[24] (Reproduced with permission from John Wiley & Sons, Inc.)**

Figure 3 is a schematic of a typical SPE cartridge that resembles a filter cartridge, except that the barrel is filled with sorbent held in place by two filters at each end of the sorbent.[20] The types of sorbents or stationary phases used in these cartridges are the same as the stationary phases commonly used for HPLC columns. The cartridges can be run individually or placed in manifolds that hold 10 or more cartridges. SPE is also carried out using SPE membranes that are particle-loaded membranes where the particles are held together within a network of polytetrafluoroethylene (PTFE) fibrils.[21] These disks come in a variety of sizes and can be used on standard extraction manifolds or used on systems designed specifically for the disk. In addition, SPE systems have been designed for very small extractions (micro-liters) by placing the sorbent in a disposable pipette tip. For high-throughput extractions, both fixed and flexible well plates that hold as many as 96 identical SPE cartridges have been designed (Figures 4 and 5).[22] The flexible well plates allow several different stationary phases to be run simultaneously reducing the time required for the development.

Many laboratories use SPE for the following reasons:[23,24] (1) SPE requires small volumes of organic solvent; typically milliliters instead of the liters required for traditional techniques like LLE.[25] Reducing the amount of organic solvent necessary to complete the extraction also reduces the risk of exposure to hazardous solvents. (2) SPE cartridges are small and well suited for field sampling.[26] (3) SPE method development can be carried out on a single SPE cartridge prior to investing in multiple cartridges or automated systems. (4) The formation of emulsion is seldom a problem in SPE. (5) SPE cartridges are inexpensive and disposable. This, combined with the reduced solvent cost makes SPE significantly less expensive than

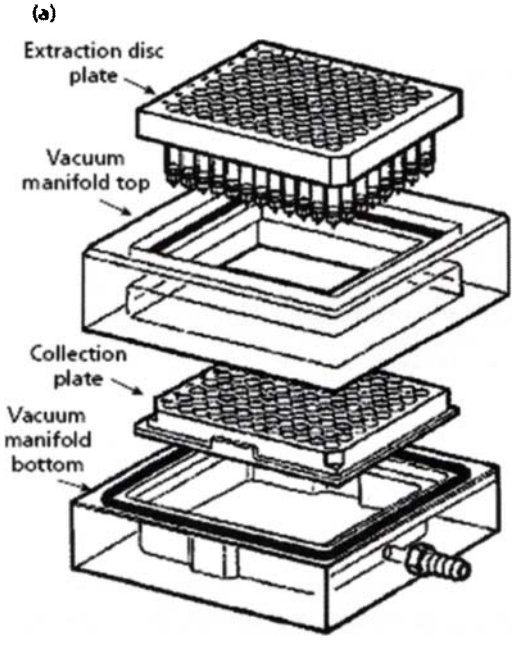

FIGURE 4 Diagram of a fixed 96-well SPE plate system.[22] (Reproduced with permission from LCGC North America.)

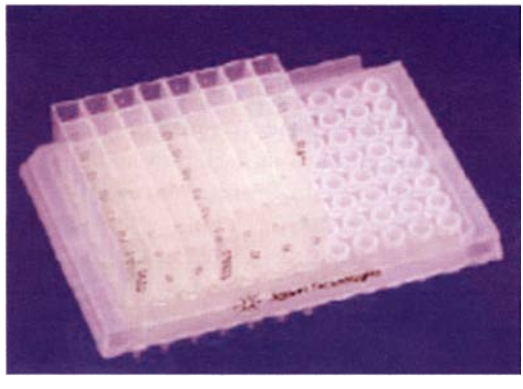

FIGURE 5 Photograph of a flexible 96-well SPE plate with removable cartridges and plug strips.[22] (Reproduced with permission from LCGC North America.)

LLE.[27] Using new cartridges for every extraction also eliminates contamination from poorly cleaned glassware. (6) SPE cartridges are reusable, like an HPLC column, if a cartridge is not overly contaminated it can be cleaned and reused. (7) Since SPE is not limited to solvents that are immiscible with water, as in LLE, more solvents can be used in SPE method development. (8) Since SPE cartridges are commercially available

in an assortment of packing materials, SPE offers a broad range of selectivity (α);[28] and (9) analysts can often find SPE methods in the application notes provided by SPE vendors[29,30] or the analyst can quickly develop a method using the basic principles of retention in HPLC.

B. Procedures and Equipment

The method for the extraction of analytes from an aqueous sample typically includes sorbent activation or conditioning, sample addition, washing, drying and elution. Figure 6 shows the typical SPE procedure schematically.[31]

1. Conditioning the surface of the sorbent material is an important step since it allows proper wetting of the sorbent surface and increases the interaction between the bonded phase on the silica sorbent and the analytes in the liquid sample matrix. Omission of this step can cause low analyte recovery. Typically, 5–10 bed volumes of strong solvent, often the elution solvent, are passed through the sorbent bed. For HPLC analysis, the organic component of the mobile phase would be an appropriate solvent. This step also removes any residual contaminants present in the sorbent that are soluble in the elution solvent. Next an intermediate solvent, typically methanol, is used to remove the conditioning solvent and prepare the sorbent for rinsing with water. The water rinse removes any excess organic and prepares the bed for the addition of the sample. If too much water is passed through the sorbent bed in this step, the bed will not remain wetted and low recoveries may be obtained.[32,33]
2. The sample can either be pushed or pulled through the sorbent bed. It is important to quantitatively transfer the sample to the reservoir. Sample volumes can range from micro-liters to liters. The analyst should determine the breakthrough volume for the analytes during method development. The breakthrough volume is the volume of sample that causes the analytes to elute from the cartridge. Once this point is reached the method is no longer quantitative. For aqueous sample the more polar the analytes, the smaller the breakthrough volume will be. One hundred percent recovery of the analytes may be possible without additional sample preparation. However, if necessary, several steps can be taken to improve the trapping efficiency of the analyte during sample addition. Since these cartridges are disposable during method development, several different stationary phases can be run simultaneously to obtain the proper selectivity. After choosing the sorbent with proper selectivity for the sample, the adsorption efficiency of the solid sorbent can be further increased by reducing the interactions of the analyte with the aqueous matrix. A common approach is to add salt such as sodium

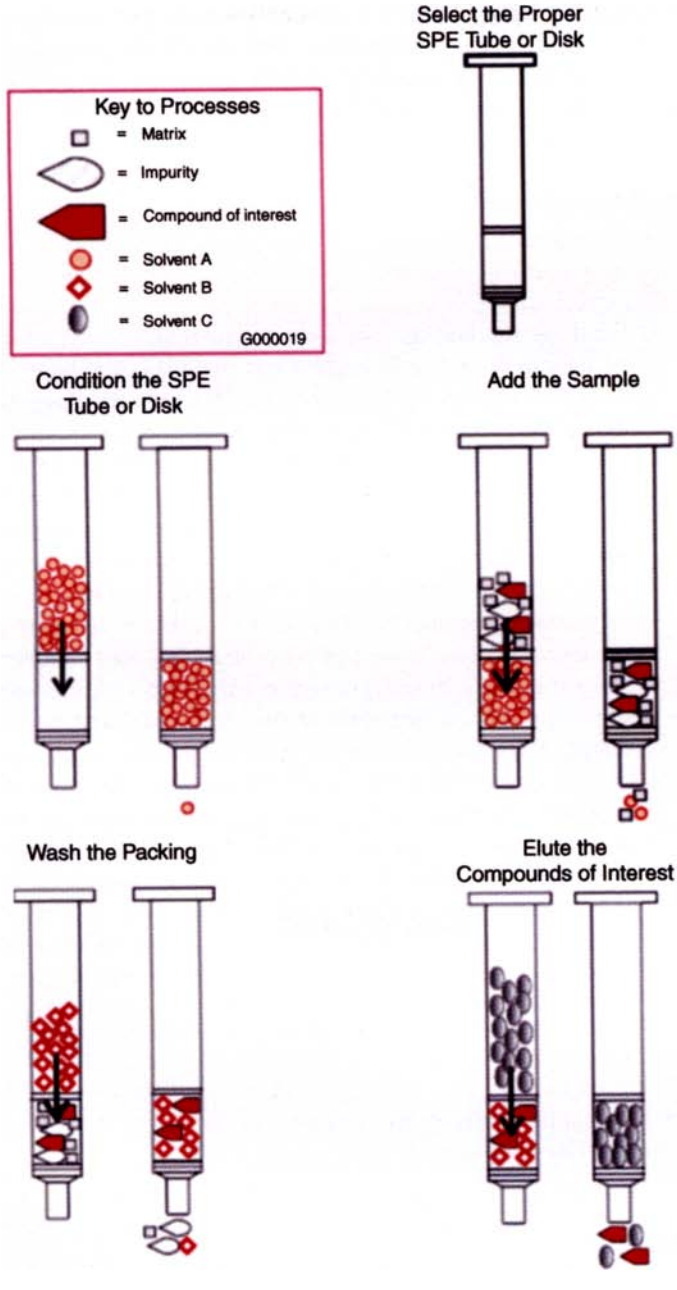

FIGURE 6 Schematic of SPE process.[31] (Reproduced with permission from Supelco, Inc.)

chloride and potassium sulfate to the sample. This works well for non-dissociating compounds. However, ionic analytes may require neutralizing by adjusting the pH of the sample. Concentrated or complex samples may require a larger surface area of sorbent to retain all of the analytes. Since extraction cartridges and disks are available in a wide range of sizes increasing the sorbent bed is not difficult. One problem that can result from passing a large volume of aqueous sample through the sorbent is bed deactivation. This can be avoided by adding 1–5% methanol to the sample to keep the bed activated.[32,33]

3. Washing the bed may be required to remove interfering contaminants and is accomplished by passing a weak solvent through the bed. The removal of contaminants without affecting the recoveries for the analytes of interest can be difficult. The washing process should be evaluated carefully to ensure that the recovery for the desired analytes is not affected by this step. Typically, the analytes should be able to withstand 20 bed volumes of wash solvent before eluting.[32,33]
4. To dry the sorbent bed water is removed by passing a stream of air or nitrogen through the bed, using a centrifuge or by placing the sorbent in a desiccator. The purpose of this step is to remove water from the sorbent and prepare it for elution of the analytes. This step may not be necessary if the elution solvent is miscible with water. Also, this step can result in the loss of volatile analytes.
5. Eluting the analytes in a small volume of strong solvent compatible with the analytical method prepares the analytes for analysis. The exact volume of elution solvent required for complete elution should be accurately determined and then the extraction method should call for a volume that ensures complete recovery during the routine use of the method. A typical approach is to perform the elution step twice using 1 μL of solvent for every milligram of sorbent.

For a detailed review of SPE and an extensive list of applications, the reader should visit SPE suppliers' websites. These sites provide many applications of SPE as a sample preparation technique for all types of chromatographic analysis along with an excellent introductory guide on SPE method development.[34–36]

VII. SOLID SAMPLES

A. Liquid Extraction

For solid samples that are either the pure compound, tablet or capsule, the sample analyzed is often a sub-sample of the powder or a composite of several tablets or capsules (often <20) with a portion prepared for injection. To extract the analytes from the solid samples and transfer to a liquid

for HPLC analysis requires a solvent extraction. There are two options: (1) directly dissolve the entire sample into an appropriate solvent or (2) pass the solvent through the sample and remove the analyte. Common examples are the shake-flask method [37] and Soxhlet extraction.[38]

Typically, the analyst has the advantage of knowing the composition of the matrix of the pharmaceutical samples to be analyzed, which will lead to the rapid development of a sample preparation method. The conventional approach to preparing less complex solid samples is to dissolve in an appropriate solvent, filter or centrifuge the sample and inject into the HPLC. The simplicity of this approach can be misleading. The key to this approach is to assure that there are no losses of the analytes of interest during this process. Sample loss can occur during sampling (from the container provided to the testing lab), grinding, transferring, dissolving, filtering or injecting the sample. All of these steps will affect the method precision and can be evaluated together by well-designed recovery studies. If acceptable recoveries are achieved then the losses from these steps are not likely to affect the method. However, if the recoveries are not acceptable then each step of the sample preparation and the solubility parameters discussed in Section II may need to be evaluated to determine the mechanism that results in the loss of analyte.

B. Shake-Flask

The shake-flask method works well when the analyte is soluble in the extraction solvent and the matrix is soluble or porous enough to allow all of the analyte to move into the solvent.[39] This technique works well for the analysis of tablets and capsules and has the advantages of eliminating the need to grind the sample. Placing whole tablets directly into the extraction flask minimizes the potential loss of analyte. Several different approaches can be employed to reduce the time required to break up the tablets such as type of shaking (shaker-box or wrist action), swirling, heating, homogenizing[40] and sonication. For tablets that dissolve too slowly for the whole tablet approach, practical grinding may be necessary. The use of mechanical or manual grinders presents the potential for loss of sample. The analyte can be adsorped to the container wall. Stratification of the ground particles may also occur, which may lead to uneven distribution of the analyte in the sample matrix. For example, the smaller particles may have a disproportionate concentration of the analyte, relative to the large particles. Therefore, it is necessary to remove a representative sample from the container.

C. Soxhlet Extraction

Soxhlet extraction is a classic technique for the transfer of analytes from solid sample into an organic solvent. This method is often used

as the accepted standard when evaluating newer techniques such as microwave-assisted extraction (MAE), accelerated solvent extraction (ASE) and supercritical fluid extraction (SFE). Soxhlet extraction is straightforward. The solid sample is placed in a porous thimble above a reservoir that holds several hundred milliliters of solvent; the solvent is heated or boiled, and as the solvent rises through a cooling jacket it condenses and seeps into the solid matrix that is supported by the porous thimble. When the thimble is full, solvent is siphoned back into the solvent reservoir and cycled through the process again. This allows the sample to be continually exposed to fresh solvent and increases extraction efficiency.[41,42] Drawbacks of this extraction technique are that extracting a single sample can take several hours or as much as 2 days and consume large amounts of solvent. However, to prepare large numbers of samples, several extractors can run simultaneously, requiring a significant amount of lab-hood space. This is particularly notable when comparing Soxhlet to either ASE or supercritical fluid extraction (SFE) which have shorter individual extraction times and are easily automated. However, automated Soxhlet extractors are available and depending on the time required to perform the extraction the most efficient and reliable approach may be to run several Soxhlet extractions simultaneously.

VIII. ADDITIONAL SAMPLE PREPARATION METHODS

The traditional approaches to sample preparation are still the most commonly used procedures for pharmaceutical analysis. However, there are several additional techniques that may be considered when developing methods. Table 5 lists several additional techniques and their applications. Most of these techniques are useful when developing HPLC methods to extract analyte from complex matrices such as natural products, animal feed, biological fluids and tissues or from products where traditional methods are not as efficient. These situations are more common in preclinical and toxicological studies than in quality control environments.

TABLE 5 Additional Sample Preparation Techniques

Technique	Applications
Accelerated solvent extraction (ASE)	Tablets, capsules, animal feed, natural products
Microwave-assisted extraction (MAE)	Tablets, capsules, animal feed, natural products
Solid-phase micro-extraction (SPME)	Biological fluids, serums, analytes in complex aqueous matrices
Supercritical fluid extraction (SFE)	Biological fluids, tablets, capsules, animal feed
Derivatization	Quality control, improved detection, Chiral separations, biological samples

A. Accelerated Solvent Extraction

ASE extracts analytes from solid matrices by passing a solvent at elevated temperatures and pressures through a solid sample. This technique has the ability to be applied to several pharmaceutical matrices such as ground or whole tablets,[43] transdermal patches,[44] animal feeds,[45] natural products,[46–48] tissue[49] and sediments.[50] There are no real restrictions on the solvents that can be used provided they are not corrosive to the extraction system; however, for HPLC analysis they should be compatible with the mobile phase used in the LC method. ASE instruments are available for single or manual operation that is more affordable and useful in labs where single runs are common such as in method development or troubleshooting. However, one main advantage of ASE is that most extractions are rapid and can be automated, which is essential for quality control labs that perform numerous analyses of solid samples. As seen in Figure 7, ASE instrumentation is composed of a pressure pump, which is used to deliver the extraction fluid to a vessel that can withstand high pressures

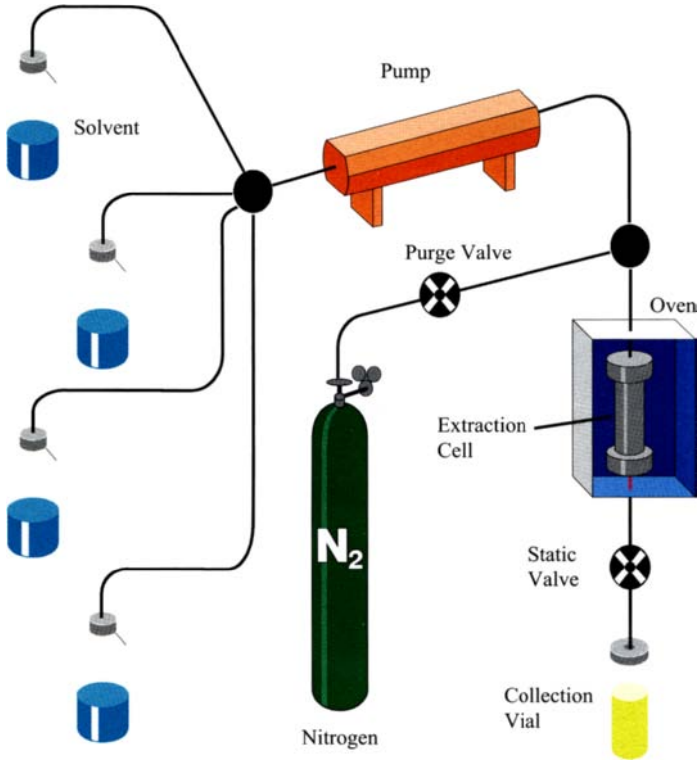

FIGURE 7 Schematic of an accelerated solvent extraction system. (Courtesy of Dionex Corporation, Salt Lake City Technical Center.[89])

and temperatures. The vessel is heated and has an automated sealing mechanism, which controls the pressure and solvent flow rate, and the extract is collected in collection vessels. The elevated temperatures increase the solvent's extraction efficiency. The rate of mass transfer and the increased pressure improves the solvent's penetration into the sample, which accelerates the rate of extraction and reduces solvent volumes and elution times.[51] The operation of the instrumentation and the extraction process are both straightforward and require little training. The extraction vessel is loaded with the solid sample (ground or whole) and then placed in the ASE instrument where the extraction process is automated. Samples that are semi-solid or that contain high amounts of moisture can be dispersed in diatomaceous earth prior to loading in the extraction vessel. The extraction can either be dynamic, static or a combination of both with multiple extraction cycles. For a static extraction, the solvent is introduced to the vessel where it is heated and pressurized. The system is allowed to stand (static extraction) and after a period of time, a nitrogen purge of the vessel flushes the solvent to a sample collection vial. The sample can then be analyzed by an appropriate method. While several parameters, such as temperature, pressure, time, number of flush cycles and solvent selection can be optimized to influence the solubility; mass transfer and desorption of the analytes from the sample matrix, the solvent choice and extraction temperature have the greatest influence on the extraction efficiency.

B. Solid-Phase Micro-Extraction

SPME is a partitioning extraction technique like LLE where the organic liquid phase is replaced by a stationary phase, a coated fused-silica fiber. This technique was developed in the early 1990s and used to extract analytes from an aqueous media by immersing the coated fiber into a liquid sample.[52] The analytes present in the liquid sample are absorbed into the organic phase where they are retained until analyzed by the appropriate technique. Due to the ease of sample introduction into a gas chromatograph, and the development of headspace SPME,[53] SPME is more commonly used for the analysis of volatile and semi-volatile analytes. However, an HPLC interface was demonstrated in 1995[54] and an SPME–HPLC interface is commercially available. SPME–HPLC has been applied to the analysis of active components in plasma,[55] serum[56] and human urine.[57]

Today, there are a variety of commercially available extraction fibers that are contained within a holder that has a mechanism for the fiber to be extended out from the needle and retracted back. This provides both protection and easy transport of the fiber phase. A schematic of the device is presented in Figure 8 and pictures of the commercially available SPME holders are presented Figure 9. The fundamental extraction process to extract analytes from a sample in a sealed vial for HPLC analysis is

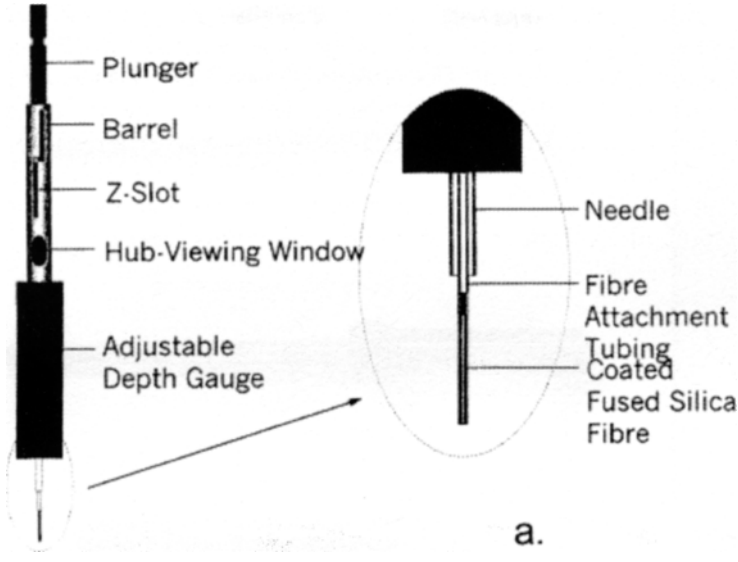

FIGURE 8 Schematic of commercial SPME device: (a) expanded view of the needle and fiber.[60] (Reproduced by permission of The Royal Society of Chemistry.)

illustrated in Figure 10 and are as follows: (1) In the extraction step, the needle containing the fiber pierces the septum of a sample vial and then the fiber is extended from the protective needle and exposed to the sample matrix. (2) Once the system is brought to equilibrium, the fiber is retracted into the syringe needle and removed from the sample vial. (3) The needle is then transferred to an SPME–HPLC interface (Figure 11) where the sample is re-extracted into a desorption solvent and injected onto the HPLC using a six-port valve.[58] As long as the analytes can be adsorbed from the sample matrix and desorbed for analysis, SPME should be possible. For a detailed discussion of the different modes of SPME (direct immersion, headspace and membrane protected extraction) and the parameters that affect the extraction efficiency such as pH, temperature, ionic strength, agitation and extraction time, the reader should refer to the text[59–61] and application guides[62,63] that have been published.

Since the introduction of SPME, the study of alternate approaches to adsorption-based micro-extraction has increased and several versions now exist. Sorbents have been placed inside of capillaries "in-tube SPME"[1–2] on stir bars, vessel walls, suspended particles and disks that are dropped directly into the sample matrix.[67]

C. Microwave-Assisted Extraction

The use of microwaves to heat the sample allows for rapid heating and extraction at elevated temperatures and pressures. MAE can be used

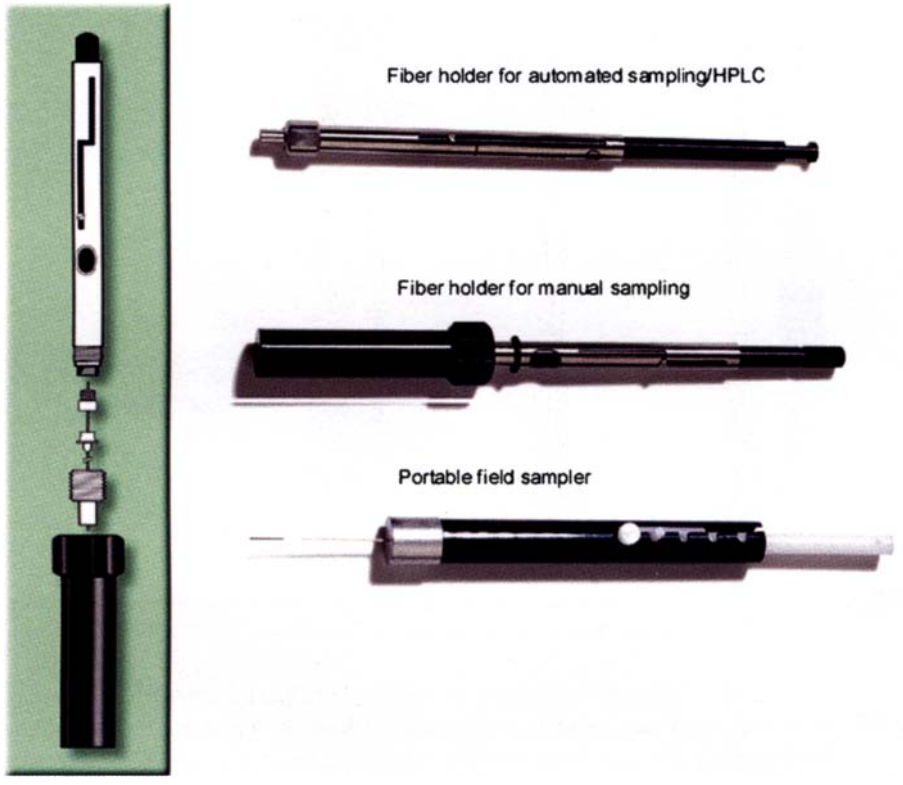

FIGURE 9 SPME fiber holder assemblies. From Supelco, Inc., Bellfonte, PA.[62] (Reproduced with permission from Supelco, Inc.)

with both microwave-absorbing solvent and non–microwave-absorbing solvent. The sample and solvents are placed in a microwave compatible container, which is sealed and is able to withstand the pressures generated during the extraction – typically, no more than a few hundred psi. The sample container is constructed of material that is chemically inert and able to withstand the elevated temperatures reached during extraction. Common materials used for MAE are composites, PTFE and quartz. When using solvents that do not absorb microwaves, the sample, which usually contains water, is heated and the hot analytes are released and retained in the solvent.[68] The extraction can be optimized by controlling the heating time, method of heating (pulsed or continuous), vessel configuration (open or closed), sample agitation, sample stirring and rate of cooling. Microwave extraction has been used for the extraction of natural products,[69] and as the heat source for Soxhlet extraction.[41]

D. Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a technique that uses a dense gas, typically carbon dioxide, which is above its critical temperature and

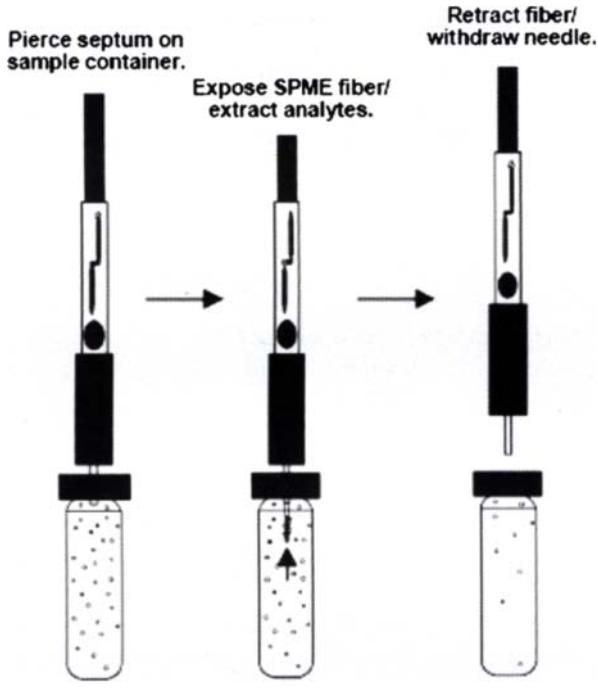

FIGURE 10 SPME extraction procedure.[58] (Reproduced with permission from Supelco, Inc.)

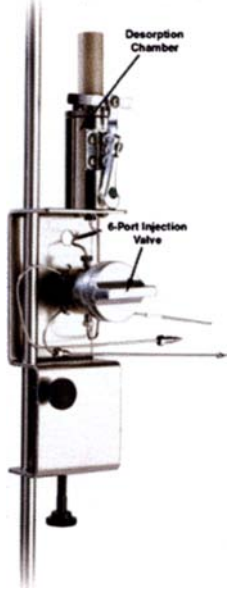

FIGURE 11 Commercially available SPME HPLC interface.[90] (Reproduced with permission from Supelco, Inc.)

pressure, to extract analytes from a solid matrix. This is possible because the dense gas or supercritical fluid has gas-like viscosity and diffusivity and densities and solvating properties that are close to those of a liquid. The majority of SFE applications have been used for the extraction of analytes from soils, agricultural products and foods followed by GC analysis. However, SFE has been used to extract analytes from plasmas,[70] urine[71] and herbs[72] for LC analysis. Supercritical fluids are capable of extracting analytes from pharmaceutical matrices such as polymers, ground tablets and natural products. The use of SFE has been decreasing over the years in part due to the growth of ASE, which employs much of the same instrumentation and methodology of SFE. Several texts have been written on SFE and provide a comprehensive review of the technique.[73–75]

E. Derivatization

Derivatization is the chemical modification of an analyte by reaction with a reagent to alter its chemical and physical properties. HPLC samples are typically derivatized to improve the detection, separation or stability of the analytes. Since derivatization increases the analysis time, the complexity of an HPLC method and its validation process, it should be used only when absolutely necessary. However, due to the effectiveness of the technique it is still widely employed and a review of the literature results in thousands of references. Also, the chemical reactions and reagents required for many derivatization applications are well established and commercially available allowing the analyst to quickly develop the appropriate method.[76] When using a derivatization kit, to ensure the completeness of reaction (quantitative), reproducibility of the reaction, product stability, production of by products and precision of any automated procedure, the derivatization reaction should be validated prior to use in a quality control lab.

A common reason to derivatize analytes in HPLC is to improve detection by adding a chromophore[77] or fluorophore[78] to an analyte that is not sufficiently detectable in its current form using UV-vis or fluorescence detection, respectively. Derivatization is also used to allow for electrochemical[79] and radioactive[80] detection and enhance mass spectral detection. The use of chemical derivatization in LC/MS can increase the sensitivity and specificity of LC/MS methods considerably for less polar compounds as well as offer additional structural information and ability to analyze volatile acids like formic acid, which are difficult to achieve by GC/MS.[81]

For the purpose of improving detection of analytes, derivatization can occur before or after separation commonly referred to as pre- or post-separation. With both having advantages and disadvantages. For development purposes pre-column derivatization can be performed

manually[82,83] or can be automated[84] and is less complicated than post-column detection since there are fewer equipment and chemical compatibility restrictions. The derivatization reactions can be preformed at the bench and do not have constraints on the solvents used or kinetics of the reaction. After derivatization, the analytes can be transferred to an LC compatible solvent and appropriate sample vial for analysis.

Post-column derivatization minimizes artifact formation and the need for a complete reaction, provided the reaction is reproducible. However, post-column derivatization requires that the reagent be compatible with the LC mobile phase and the use of a reaction detector. Many classes of compounds commonly analyzed in pharmaceutical analysis such as steroids, amines, antibiotics and barbiturates can be derivatized.[85]

Derivatization of analytes can also be used to improve the separation of chiral analytes that are common in pharmaceutical industry. Enantiomers can be resolved using conventional separation techniques by first forming diastereoisomers via deriviatization with enantiomeric chiral agents.[86] Derivatization for the analysis of chiral compounds has been applied to peptidic antibiotics, toxins and pharmaceutical amino acids.[87] The reproducibility of this technique is also acceptable for application to quality control monitoring.[88]

IX. CONCLUSIONS

In pharmaceutical analysis, sample preparation is the main area in which the analyst will manipulate samples and have a direct effect on quantitative analysis. Since modern instrumentation is designed in many ways, to self-check in order to ensure that it is working properly, the main source of laboratory deviations results from the sample preparation scheme and how effectively it is applied by the analyst. All sample preparation methods have their theoretical basis in chemical equilibrium, so an understanding of the fundamentals of chemical equilibrium is critical for developing, troubleshooting and investigating pharmaceutical methods. Finally, sample preparation is greatly affected by the proper use of glassware and equipment. The errors associated with improper use are often subtle and not easily diagnosed.

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9

INSTRUMENT QUALIFICATION AND SOFTWARE VALIDATION

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HPLC Method Development for Pharmaceuticals
S. Ahuja and H. Rasmussen, editors.

ABSTRACT

The intent of this chapter is to provide readers from different backgrounds (pharmaceutical scientists, quality assurance (QA) personnel, and qualification personnel) an improved understanding about which laws and regulations apply to the pharmaceutical industry and how they impact equipment qualification (EQ) efforts. In the first part, the different elements that cause competing interpretations of requirements and confusion about what exactly is required to achieve compliance with regulations that pertain to equipment qualification are discussed. The second part of the chapter will discuss such widely used terms as calibration, qualification, and validation, while shedding light on what differences and similarities exist between equipment qualification, validating a method, and performing system suitability tests. In the third part of the chapter, a model is discussed, which can be used to ensure that qualification activities occur in a systematic organized manner and enables the reader to implement a scientifically sound decision-making process. In the fourth part, an in-depth case study will focus on the critical aspects of different high-performance liquid chromatograph (HPLC) modules (solvent delivery system, detector, column compartment, and an injector/autosampler) from a qualification perspective. Most HPLCs comprise these modules as well as software and firmware components. In the final part, the content will be summarized and conclusions will be formulated enabling the reader to implement the model in a cost-effective manner.

I. INTRODUCTION

Equipment qualification programs implemented by the pharmaceutical industry vary considerably in the responsibilities assigned to the groups that perform and the procedures that govern the qualification activities. These variations are caused by:

- the absence of clear authoritative guidance, and
- the lack of detail in internationally recognized quality standards. (Both the guidance documents and the quality standards are deliberately written in general terms to be widely applicable.)

In addition, many factors lead to competing interpretations of the requirements and/or confusion about what exactly is required and how to achieve compliance, including

- voluntary standards,
- different terminologies used and different levels of support provided by suppliers,
- varying levels of awareness of the Food and Drug Administrations (FDAs) and their European counterparts Good Manufacturing Practices (GMPs), and
- varying levels of experience on how to implement an effective equipment qualification program.

The main objective of the regulations is to ensure patient safety, achieved by producing a high-quality product, which in turn, is attained

by the creation of reliable data. The latter is the main focus of the equipment qualification programs; they provide documented evidence that a specific piece of equipment is suitable for its intended use and, therefore, creates reliable data. However, the applicable regulations (21CFR58, 21CFR11, 21CFR210, 21CFR211, Annexes 11, 15, and 18) and most guidance documents do not provide any details on how to validate processes and qualify instruments/equipment. Since the drug product is defined in term of its physical, chemical, and performance characteristics, which are translated into specifications that make quality control possible as defined in the Research and Development (R&D) stage, the regulations do not differentiate between a quality control lab in a production environment and a R&D laboratory – these same standards apply for both.[1] Although the FDA regulations do not mention the Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ), these terms are widely used and described in, quite often very lengthy, procedures and guidance documents throughout the pharmaceutical industry. As a result of these (lengthy) procedures, the actual value of qualification is diminished and the focus has shifted from the drug product to the "paper product" many pharmaceutical companies produce. In the following chapters, the definitions of each of the aforementioned phases (DQ, IQ, OQ, and PQ), and the activities that *typically* comprise each phase of the qualification will be described. It is important to highlight that it is not important whether a certain activity is categorized as a DQ, IQ, OQ, or PQ activity, but it is critical that the activity is performed or documentation provided for not performing the activity.

II. DEFINITIONS

A. Equipment

A device or collection of devices, including their firmware and computerized controllers used to perform a mechanical process or produce a result. The term equipment encompasses instruments, e.g. blenders, high-performance liquid chromatographs (HPLCs), (tapped) density meters, particle counters, viscometers, etc.

B. Instrument

A device (chemical, electrical, hydraulic, magnetic, mechanical, optical, pneumatic) used to test, observe, measure, monitor, alter, generate, record, calibrate, manage, or control physical properties, movements, or other characteristics.[2] Examples include timers, balances, pressure gauges, chart recorders, refrigerators, water baths, voltmeters, tachometers, temperature controllers, etc.

C. Standardization

The process of assigning a value to one standard based on another standard. For example, thermometers, sieves, and pH meters are standardized. The terms standardization and calibration are often incorrectly used interchangeably. Standardization of equipment assures precision,[3] the closeness of agreement (degree of scatter) between a series of measurements; while calibration assures accuracy,[3] the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.

D. Calibration

Calibration is defined by the EU regulators[4] as "the demonstration that a particular instrument or device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements" and by ANSI/ISO[5] as "the set of operations that establish, under specified conditions, the relationship between values indicated by a measuring instrument or measuring system, or values represented by material measure and the corresponding values of the measurand." Calibration is performed on instruments that are used to determine an absolute value to assure accuracy of this value. For example, equipment that requires wavelength accuracy (radiation sources, detectors), balances, and temperature recording devices are calibrated. Calibration must be performed by utilizing references standards and materials traceable to national or international standards (NIST, ASTM, etc.) to ensure accuracy of the data produced.

E. Verification

21CFR820 defines verification as "a means of confirming by examination and provision of objective evidence that specified requirements have been fulfilled."[5]

F. Qualification

The EU regulators[4] define qualification as the act of proving and documenting that equipment or ancillary systems are properly installed, work correctly, and actually lead to the expected results. Qualification is part of validation, but the individual qualification steps alone do not constitute process validation. The terms "qualification" and "validation" are often erroneously used interchangeable. The term qualification is reserved for equipment, while the term validation pertains to processes, methods, and software. Equipment qualification (EQ) provides

documented evidence that the equipment is suitable for its intended use, improves confidence that the data produced by qualified equipment is reliable, and provides the basis for all subsequent analytical work (including method validation). Once equipment comprising different modules has been qualified, it must be treated as one unit. For example, a detector of a qualified HPLC cannot be transferred from one qualified system to another. The firmware versions of both units could be different and the exact impact of minor difference in firmware can only be assessed by the manufacturer/supplier of the equipment.

G. Validation

Outside FDA regulated industries, validation is defined as the act, process, or instance of validating and the determination of the degree of validity of a measuring device. In turn, valid is defined as having legal efficacy or force; executed with the proper legal authority and formalities, and it implies being supported by objective truth or generally accepted authority.[6] The FDA defines validation as ". . . establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specification . . ."[1] and in 21CFR820 as ". . . establishing by objective evidence that device specifications conform with user needs and intended use(s) . . . ." Annex 18[4] expands these definitions to: "A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting pre-determined acceptance criteria." While Annex 15 of the *EU Guide to Good Manufacturing Practices*[7] provides a more detailed definition for (process) validation ". . . The documented evidence that the process, operated within established parameters, can effectively and reproducibly produce a medicinal product meeting its predetermined specifications and quality attributes . . . ." The EU regulators (section 12.30 of Annex 18)[4] underline that before starting process validation activities, appropriate qualification of critical equipment and ancillary systems should be completed. Validation provides documented evidence that a process, method, or software package is suitable for its intended use, that a product has been produced in accordance with legal formalities, or provides data that is legally valid. The legal formalities described in the applicable regulations are translated into standard operating procedures (SOPs). As described above, validation pertains to processes, methods, and software applications. It should be noted that the FDA proposed to amend the cGMPs to include a section (211.220) outlining the regulatory requirements for process validation.[8] The European Authorities[9] require that where a computerized system replaces a manual operation, consideration should be given to the risk of losing aspects of the previous system, which could result from reducing the involvement of

operators. The extent of validation necessary will depend on a number of factors including:

- the use to which the system is to be put,
- whether the validation is to be prospective or retrospective, and
- whether or not novel elements are incorporated.

Validation should be considered as part of the complete life cycle of a computer system. This cycle includes the following stages in chronological order:

- planning
- specification
- programming
- testing
- commissioning
- documentation
- operation
- monitoring
- modifying

Annex 11,[9] which contains more stringent requirements than its counterpart in the US 21CFR11,[10] specifies the following requirements for the qualification of computerized systems:

(a) The computerized system should be installed in a suitable environment.
(b) An up-to-date description of the principles, objectives, security measures, scope of the system, the main features of the way in which the computer is used, and how it interacts with other systems and procedures.
(c) Software is seen as critical component of a computerized system. Hence the *user*, not the company that developed, of the software should take all reasonable steps to ensure that it has been produced in accordance with a system of quality assurance.
(d) The system includes, where appropriate, built-in checks of the correct entry and processing of data.
(e) Before a system using a computer is brought into use, it should be thoroughly tested and confirmed as being capable of achieving the desired results.
(f) Suitable methods of deterring unauthorized entry of data include the use of keys, pass cards, personal codes, and restricted access to computer terminals. There should be a defined procedure for the issue, cancellation, and alteration of authorization to enter and amend data, including the changing of personal passwords.

Consideration should be given to systems allowing for recording of attempts to access by unauthorized persons.

(g) When critical data are being entered manually (for example, the weight and batch number of an ingredient during dispensing), there should be an additional check on the accuracy of the record that is made.

(h) The system should record the identity of operators entering or confirming critical data. Authority to amend entered data should be restricted to nominated persons. Any alteration to an entry of critical data should be authorized and recorded with the reason for the change. Consideration should be given to building into the system the creation of a complete record of all entries and amendments (an "audit trail").

(i) Alterations to a system or to a computer program should only be made in accordance with a defined procedure, which should include provision for validating, checking, approving, and implementing the change. Such an alteration should only be implemented with the agreement of the person responsible for the part of the system concerned, and the alteration should be recorded. Every significant modification should be validated.

(j) For quality auditing purposes, it should be possible to obtain clear printed copies of electronically stored data.

(k) Data should be secured by physical or electronic means against willful or accidental damage. Stored data should be checked for accessibility, durability, and accuracy. If changes are proposed to the computer equipment or its programs, the above-mentioned checks should be performed at a frequency appropriate to the storage medium being used.

(l) Data should be protected by backing-up at regular intervals. Back-up data should be stored as long as necessary at a separate and secure location.

(m) There should be available adequate alternative arrangements for systems, which need to be operated in the event of a breakdown. The time required to bring the alternative arrangements into use should be related to the possible urgency of the need to use them. For example, information required to effect a recall must be available at short notice.

(n) The procedures to be followed if the system fails or breaks down should be defined and validated. Any failures and remedial action taken should be recorded.

(o) A procedure should be established to record and analyze errors and to enable corrective action to be taken.

(p) When outside agencies are used to provide a computer service, there should be a formal agreement including a clear statement of the responsibilities of that outside agency.

In the US, similar requirements are described in Title 21 Part 11 (21CFR11 – "Part 11,"[10] which outlines the requirements for Electronic Records and Signatures), and 21CFR211.68[11] (Automatic, Mechanical, and Electronic Equipment). The regulatory authorities recognize that commercially available software that has been qualified does not require the same level of testing[4] and that when the software is developed by someone other than the device manufacturer (e.g. off-the-shelf software), the software developer may not be directly responsible for compliance with regulations. In that case, the party with regulatory responsibility needs to assess the adequacy of the off-the-shelf software developer's activities and determine what additional efforts are needed to establish that the software is validated for its intended use. When purchasing "off-the-shelf" software, they must ensure that it will perform as intended in their chosen application.[12]

H. Method Validation

The EU regulators specify (section 12.82 of Annex 18)[4] that appropriate qualification of analytical equipment should be considered before starting validation of analytical methods. Based on the definition of validation, the definition[1] of method validation is as follows: ". . . establishing documented evidence which provides a high degree of assurance that a method will consistently produce results that meets predetermined specifications . . . ." Accuracy, precision, sensitivity, specificity, repeatability, linearity, robustness, ruggedness, and stability are the parameters of interest during method validation.[3,13,14] The objective of method validation is to demonstrate its suitability for its intended use.[3,14] 21CFR211.194 (a)[15] requires that test methods used in the testing of sample meet proper standards of accuracy and reliability, this requirement has been incorporated in the United States Pharmacopoeia (USP).[13] Method validation does for methods what equipment qualification does for equipment by providing confidence that the method and equipment, respectively meets the predetermined requirements and is therefore suitable for its intended use.

It should be noted that the FDA proposed to amend the cGMPs to include a section (211.222) outlining additional regulatory requirements for method validation.[8] The results generated by a validated method on qualified equipment can be considered as reliable. The concepts that can also be deployed in equipment qualification are discussed below.

1. Accuracy

Accuracy expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found. ICH recommends that a minimum of nine determinations over a minimum of three concentration levels covering

the entire range should be assessed to determine accuracy.[3,13,14] Accuracy of modules is often a critical parameter in equipment qualification due to the potential impact on the reliability of the data generated by the equipment. Some examples are spectrophotometric equipment (wavelength accuracy), viscometers (speed and temperature accuracy), particle counters (flow rate accuracy), and density measurements (temperature accuracy).

2. Precision

Precision is the degree of agreement among individual test results when the method/equipment is applied repeatedly to multiple samplings of a homogeneous sample/standard.[3,13,14] Precision is usually expressed as relative standard deviation (%RSD) (=coefficient of variation). It may be a measure of reproducibility (which expressed precision between labs) or repeatability (same procedure within the same lab).[3,14] ICH recommends that repeatability is demonstrated utilizing a minimum of nine determinations covering the specified range for the procedure (e.g. three concentrations/three replications each) or a minimum of six determinations at 100% of the test concentration.[14] The ability of equipment to repeatedly generate reliable data will also be demonstrated during equipment qualification.

3. Linearity and Range

Linearity is the ability (within a given range) to obtain an output that is directly proportional to the input. Linearity should be evaluated over the range of the analytical procedure and equipment, ICH recommends a minimum of five points to demonstrate linearity by means of statistical evaluation (correlation coefficient) of the data. The range is the interval between the upper and lower limits for a parameter (including these upper and lower limits) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.[3,13,14] As with accuracy and precision, the same concepts that apply for method validation can be deployed to demonstrate, for example, detector linearity and tests conducted to provide documented evidence that a piece of equipment is suitable to be used to determine an analytical parameter. The upper and lower limits of the qualification range are required to be included in the test.

I. System Suitability

System suitability testing (SST) is an integral part of many analytical procedures. It is based on the concept that the equipment, electronics, analytical operations, and sample to be analyzed constitute an integral system that can be evaluated as such.[14] As a direct result of this, whenever a significant change is made, SST should be successfully performed

prior to analyzing samples. SST, EQ, and method validation share a common goal, ascertaining suitability of the equipment/method for its intended use. EQ and method validation activities differ from SST as they are not performed at the time of the test or even concurrently with test samples. SST is different from PQ testing because it deals with variations in analytical conditions[13,16] – for HPLCs these variations can be caused by differences in the mobile phase (pH and composition), column (different lot or supplier), reagents (different lot/supplier), and/or equipment related variations (flow rate, temperature, signal-to-noise ratio of detectors, and injector precision), which can lead to, but are not limited to, differences in peak sharpness and retention times of the component of interest. EQ only focuses on the characteristics of the equipment: temperature, injection volume, flow rate, signal-to-noise ratio of detector, and wavelength accuracy. Another difference is that PQ testing is intended to *simulate* conditions under routine operation, while SST is performed during routine use of the equipment. Therefore, SST is supplemental to PQ tests in the sense that it assures acceptable performance at the time of sample analysis, but cannot replace PQ testing. In addition, SST is mostly performed on chromatographic equipment, while PQ tests are performed on a wide variety of equipment.

III. QUALIFICATION MODEL

President Roosevelt described the objective for enacting bill S.5, an amendment to the 1906 Food and Drug Act, the predecessor of the 1936 FD&C Act, as follows: ". . . Every enterprise in the United States should be able to adhere to the simple principle of honesty without fear of penalty on that account. Honesty ought to be the best policy, not only for one individual or one enterprise but for every individual and every enterprise in the nation. No honest enterpriser need fear that because of the passage of such a measure he will be unfairly treated. He would be asked to do no more than he now holds himself out to do. It would merely make certain that those who are less scrupulous than I know most of our producers to be cannot force their more honest competitors into dishonorable ways. The great majority of those engaged in the trade in food and drugs do not need regulation. They observe the spirit as well as the letter of existing law. Present legislation ought to be directed primarily toward a small minority of evaders and chiselers. At the same time even-handed regulation will not only outlaw the bad practices of the few but will also protect the many from unscrupulous competition. It will, besides, provide a bulwark of consumer confidence throughout the business world . . . ."[17] Although the Code of Federal Regulations (CFRs) governing the GMP, GLP, and GCP activities do not use the terms Design Qualification, Installation Qualification, Operational

Qualification, and Performance Qualification, these terms are widely used in the pharmaceutical industry. While the FDA guidance documents on process and software validation[1,12] only define IQ activities for EQ and PQ activities for products and processes, and IQ, OQ, and PQ activities for software validation, the European Commission has defined all four phases in the *EU Guide to Good Manufacturing Practices*[4,7]. All four phases and terms are widely used in the equipment qualification programs in the pharmaceutical industry. As a result of this, there is no consensus on which activities are included in which phase of the equipment qualification program; nevertheless, an overview will be provided on the activities *typically* performed during each of these phases. It is critical that the required activities are performed, in which phase the activities reside is of lesser importance. In general, the following steps can be identified during the qualification process:

- Development of the qualification plan, which outlines the roles and responsibilities of all parties involved in the qualification process and the extent and rigor of qualification required for the equipment that is being qualified.
- Development of a qualification protocol with describes the scope of the qualification activities, the intended use of the equipment, the critical parameters, required test steps and test tools, and the acceptance criteria.
- Execution of the qualification protocol. In this phase, the actual results are documented. Verification that these results meet the predetermined acceptance criteria should be established and any failing results should be investigated.
- A summary report is generated in which the results of the qualification activities are documented.

To facilitate a cost/time-effective qualification program, it is advisable that the wide variety of equipment available in most laboratories is divided into different categories. This categorization allows for differentiation between simple and complex equipment based on the level of qualification required for each type of equipment. A common differentiation is equipment that are controlled by a computer (computerized systems), usually connected to a Laboratory Information Management System (LIMS) on which the generated data is stored and processed, and equipment that are neither controlled by a computer nor connected to a LIMS. Guidance on how to categorize instruments is available in the Good Automated Manufacturing Practices (GAMP) – guide for validation of automated systems, developed by ISPE's GAMP Forum.[18] If the supplier provides IQ, OQ, and PQ documentation, the end-user should verify that this documentation meets the requirements described in their quality system, that tests span the entire (intended) operational range, and that the acceptance criteria are acceptable.

A. DQ

The European Commission defines design qualification as the documented verification that the proposed design of the facilities, systems, and equipment is suitable for the intended purpose.[4,7] Since neither the FDA's Guidance document on software nor its guidance on process validation[1,12] mention design qualification, Annexes 15 and 18[4,7] are the only authority documents available that indicate design qualification is the first step to demonstrate that the design of facilities, systems, or equipment complies with GMP requirements. This pre-installation phase will lead to the selection of equipment and supplier(s) that meet predetermined operational and functional requirements. These requirements can pertain to both the equipment and the supplier. Examples are the accuracy, repeatability, and/or stability of critical parameters/components within the intended operating range of the equipment, required utilities, estimated price of consumables over the lifecycle of the equipment, availability and quality of documentation (manuals, SOP's qualification documents), health, safety and environmental recommendations, ease of operation, recommended calibration/preventative maintenance frequency, compatibility with other equipment, support, service and training programs implemented by the supplier, pre-existing agreements with preferred suppliers, past experiences and interactions, reputation of the supplier within the industry, geographical proximity, and the maturity of the equipment. The majority of equipment in FDA-regulated laboratories are commercial off-the-shelf (COTS) products. As a result of this, design qualification is looked upon as an activity that should be performed by the developer and/or manufacturer. Unfortunately, not all developers and/or manufacturers are familiar with the requirements set forth by the regulatory authorities. Hence, pharmaceutical companies, as end-users, should verify that the supplier has a quality system in place that describes all aspects of development, production, and testing of the equipment, including willingness of the supplier to allow authorities to access source codes, training/qualifications of personnel involved, change control, and compliant handling. Once a vendor audit has confirmed the existence of a suitable quality system, usually based on ISO guidelines, the end-user can refer to the supplier's quality system and does not need to repeat all aspects of DQ.

B. IQ: Verification and Documenting

The European[4,7] and US regulators,[1] respectively, have defined the IQ phase as ". . . The documented verification that the facilities, systems and equipment, as installed or modified, comply with the approved design and the manufacturer's recommendations . . ."[4,7] and as ". . . Establishing confidence that process equipment and ancillary systems are capable of consistently operating within established limits and tolerances . . . ."[1] The

EU definition clearly links the IQ phase to the previous phase (DQ) by referring to the operational and functional requirements defined in the DQ phase and highlights that the instrument should be installed as per the manufacturer's recommendation, while the US definition focuses on the capability of meeting operational limits and tolerances that will be tested in the next phase (OQ). Please also note that the EU regulators require an IQ to be performed on modified facilities, systems, and equipment, while the US regulators are less specific, they require that a system is in place that assures timely re-validation.[1] The main objective of the installation qualification activities is to demonstrate that the equipment (including software) received by the supplier meets the specification defined in the DQ, the equipment is installed in a suitable environment in accordance with the supplier's procedures, and the documentation pertaining to the system is accurate and complete. To assure the aforementioned objectives are attained, the following activities are conducted during installation qualification:

(a) Verification that received equipment, piping, services, and instrumentation are installed as depicted on engineering drawings, installation in a suitable environment occurred in accordance with the supplier's procedure, and all installation specifications are met.[1,7,19–21] If the environmental specification provided by the supplier are within the specifications that are considered to be standard for a controlled laboratory environment, they do not require separate testing. For example, the manual indicates that the equipment can be installed in the following environment: 40–90°F with a %RH of 0–90%, since both the temperature and %RH specification fall well within the ranges required by the supplier, no additional testing is required. Instead, a statement in the IQ indicating that the conditions in the laboratory are within the ranges specified by the supplier suffices.

(b) Verification of materials of construction: reports of composition and material ratings should be reviewed for suitability and retained for reference.[7,20,21]

(c) Availability of required utilities and compliance of utility services with design specifications.[20]

(d) Collection, cataloging of, review and storage of documentation pertaining to the equipment (including purchase orders, filter certification (if applicable) manuals, master computer disks, health and safety recommendations, supplier operating and working instructions, calibration, and maintenance requirements).[1,7,20,21] The system's documentation is reviewed to assure it is accurate and complete. SOPs describing operation, maintenance, and calibration will be developed based on the documentation provided by the supplier. Cataloging of the

system documentation assures that the system documentation is controlled.

(e) Identification of critical equipment features that could affect the product.[1]

(f) Verification that all software associated with the equipment are identified, have the correct name, file revision number, and size.

(g) Verification of calibration status of components that require calibration.[1,20]

(h) Detailed description of the equipment: documenting internal configuration of the equipment, diagrams depicting interfaces with other modules/systems.[20] Typically, the manufacturer, model number, serial number of each component, and the soft- and firmware versions are recorded.

(i) For tracking purposes, the equipment is assigned a unique ID#, entered into the company's inventory, and placed on a maintenance/calibration schedule.

(j) An equipment-specific logbook is issued for the equipment, in which the qualification activities and other critical activities conducted on the equipment are documented.

(k) Identification (Title, DocumentID, version number, and implementation or effective date) of the SOPs describing the operation, maintenance, and calibration of the equipment.

(l) A successful installation is demonstrated by running an internal diagnostics test (if available). This function can also be used to verify that modular systems are correctly connected to each other.

The activities associated with the physical installation of complex equipment are usually done by the supplier, while the remaining activities are performed by the end-user. If the supplier provides IQ, OQ, and PQ documentation, the end-user should, as indicated by 21CFR211.160 (a):[22]

- review these documents to assure that this documentation meets the requirements described in their quality system,
- test the entire intended operational range, and
- ensure that the acceptance criteria are acceptable.

Upon successful completion of the IQ, the next phase of the qualification can be initiated.

C. OQ: Testing

As indicated by the FDA,[1] equipment should be evaluated and tested to verify that it is capable of operating satisfactorily within required operating limits and it is usually insufficient to rely solely on representations

provided by the supplier, or upon previous experiences with similar equipment.

The FDA has not defined OQ in the guidelines on process and software validation,[1,12] the only authoritative source that provides a definition in the US is the USP.[19] OQ is defined in the USP as "the process by which it is demonstrated and documented that the instrument performs according to specifications, and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, major repair, etc. . . . ." EU regulators[4,7] have defined OQ as "The documented verification that the facilities, systems, and equipment, as installed or modified, perform as intended throughout the anticipated operating ranges." Annex 15[7] states that after successful completion of OQ testing, finalization of calibration, operating and cleaning procedures, operator training and preventative maintenance requirements can be initiated. It also permits formal "release" of the facilities, systems, and equipment. Activities that are usually conducted during OQ are as follows:

(a) Tests that have been developed from knowledge of processes, systems, and equipment.[7]
(b) Tests to include a condition or a set of conditions encompassing upper and lower operating limits, sometimes referred to as "worst case" conditions.[7] Examples are temperature, timer, and wavelength accuracy tests, and linearity testing (spectrophotometers).
(c) Tests that verify the structural integrity of the equipment. Examples are leak tests (GC, HPLC, isolators, etc.).[20]
(d) Tests and inspection designed to verify that the equipment, system alerts, and controls are operating reliably and that appropriate alert and action levels are established.[20,21] Examples are temperature alarm settings in freezers, refrigerators and incubators, humidity/temperature alarms in stability chambers, and pressure alarms in isolators and HPLCs.
(e) Tests that verify that the equipment operates within target specification.[19] This includes software tests pertaining to the storage, and backup of data generated by the system.
(f) Tests that verify the calibration status of the equipment.
(g) Verification of accuracy and completeness of SOPs describing operation, maintenance, calibration, and training[7] and existence of an equipment logbook.

OQ tests should be based on sounds scientific principles, and should be designed in such a way that they are able to detect anomalies.

Although the intent of OQ testing is to verify that the equipment performs as intended over user defined anticipated operating ranges, often the ranges and associated acceptance criteria are transcribed from the supplier's manual.

Often this is not the most cost- and time-efficient approach, OQ testing should only verify that equipment operates as expected within the anticipated operating range, i.e. if a density meter is able to accurately measure densities up to 4 g/cm^3 but the user only produces products with a density up to ca. 2.5 g/cm^3, it suffices to verify the accuracy of the density measurements of up to 2.5 g/cm^3. If the user later decides to expand the operating range, additional testing is needed to demonstrate the equipment continues to operate within the acceptance criteria defined by the user. A similar situation can occur with the acceptance criteria defined by the supplier, i.e. the supplier of a polarimeter states that the temperature accuracy of the equipment is 0.3°C, while the applicable pharmacopoeia (USP28 ⟨781⟩, and EP5.02 2.2.7) requires a temperature accuracy of ±0.5°C. The user should always verify whether the ranges and specifications associated with critical parameters provided by the supplier are aligned with the specifications defined by the user. System suitability and other routine tests cannot replace OQ testing, since OQ tests are designed to verify whether the equipment operates as intended over the complete operation range, while SST and other routine tests only verify some parameters over a specific part of the operating range. The main difference between OQ testing and PQ is best observed when applied to modular systems; OQ tests are modular, they verify whether a specific module complies with the predetermined tolerances, while PQ tests tend to have a more holistic philosophy by verifying whether the entire system operates as expected during routine use. As a result of this, OQ tests often are more rigorous and detailed than PQ tests. It is recommended that after repair of a certain module, the OQ tests pertaining to this module are performed. If a module has the ability to perform a self-diagnostic, this test alleviates the need to do additional testing.

D. PQ

Annexes 15 and 18[4,7] define PQ as "The documented verification that the facilities, systems and equipment, as connected together, can perform effectively and reproducibly, based on the approved process method and product specification." As pointed out earlier, this definition implies that PQ tests have a holistic character. The FDA[1] makes the distinction between product and process performance qualification and defines PQ as "Establishing confidence through appropriate testing that the finished product produced by a specified process(es) meets all release requirements for functionality and safety," respectively, establishing confidence that the process is effective and reproducible. Since both the European and American authorities specifically mention that evidentiary support for reproducibility of the equipment/process must be available,

PQ tests are to be repeated a sufficient number of times to assure reliable and meaningful results.[1]

As described earlier, ICH recommends that repeatability is demonstrated utilizing a minimum of nine determinations covering the operational range (e.g. three different flow rates/three replications each or a minimum of six determinations at the upper or lower limits of the operational range).[14] As indicated by the EU GMPs,[7] PQ starts after successful completion of both IQ and OQ testing. Activities that are usually conducted during PQ are as follows:

(a) Tests to include a condition or a set of conditions encompassing upper and lower operating limits, sometimes referred to as "worst case."[1]
(b) Tests, using production materials, qualified substitutes or simulated product, that have been developed from knowledge of the process and the facilities, systems or equipment.[7]
(c) Tests to include a condition or set of conditions encompassing upper and lower operating limits.[7]
(d) Tests that verify the efficacy of sterilization cycles[20] (cleaning validation).
(e) Verify that procedures describing calibration, maintenance, operation, and training are implemented. In the previous phases, it was not required that these procedures were approved, effective, and implemented, but in this phase routine operation is simulated and the procedures should be implemented.

Some PQ tests can resemble OQ tests. This is especially the case for simpler (non-modular) equipment, where the two different phases blend together. In addition, it is often more efficient and practical to perform holistic reproducibility and linearity tests, which cause the boundaries between OQ and PQ tests to fade. Since PQ tests are often less rigorous than OQ tests, the tolerances associated with PQ test do not have to be identical to those used in the OQ tests. Due to the less rigorous testing required in this phase and the objective of demonstrating that the equipment performs as expected during routine use, PQ tests are executed more frequently than OQ tests. Since PQ tests are performed more frequently, they can be used to track the equipment's performance during the equipment's lifecycle, which can be used to determine the frequency of preventative-maintenance programs. The PQ tests can be performed at fixed intervals (every 6 months, every 12 months) or prior to use of the equipment (e.g. pH meters and balances). For most chromatographic equipment, PQ tests are performed every 12 months, to assure reliable data are produced. System suitability tests are run immediately before running samples. As described earlier, these SSTs are supplemental to PQ tests.

E. Change Control

References to change control can be found in both the EU[4,7,9] and US regulations[11] (21CFR211.68). Annex 15[7] defines change control as "a formal system by which qualified representatives of appropriate disciplines review proposed or actual changes that might affect the validated status of facilities, systems, equipment or processes. The intent is to determine the need for action that would ensure and document that the system is maintained in a validated state." Annex 11[9] dealing with computerized systems states that alterations to a system or to a computer program should only be made in accordance with a defined procedure which should include provision for validating, checking, approving, and implementing the change. Such an alteration should only be implemented with the agreement of the person responsible for the part of the system concerned, and the alteration should be recorded. Every significant modification should be validated. Annex 15[7] requires that written procedures should be in place to describe the actions to be taken if a change is proposed to a starting material, product component, process equipment, process environment (or site), method of production or testing, or any other change that may affect product quality or reproducibility of the process. Change control procedures should ensure that sufficient supporting data are generated to demonstrate that the revised process will result in a product of the desired quality, consistent with the approved specifications. All changes that may affect product quality or reproducibility of the process should be formally requested, documented, and accepted. The likely impact of the change of facilities, systems, and equipment on the product should be evaluated, including risk analysis. The need for, and the extent of, re-qualification and re-validation should be determined. Annex 18[4] and the US regulations[11] are both less detailed and require "changes" to be recorded. The FDA's *Guideline on General Principles of Process Validation*[1] also indicates that re-validation is required after a change has been implemented in a process or equipment that could have an impact on the effectiveness or characteristics of the product. The extent of re-validation depends on the nature of the change and its impact on the product.

In addition, the USP[19,21] recommends that when changes are made, their impact should be evaluated and *relevant* OQ tests should be performed. The extent of re-qualification depends on the significance of the change. For modular equipment, a cost-effective change control program will determine which OQ tests are relevant based on a risk analysis and will re-qualify the module that was affected in lieu of re-qualifying the entire equipment. For example, if a change was made to the pump system of a HPLC to stop a leak, only OQ tests pertaining to the pump system should be performed. Examples of significant changes are relocation or movement, modifications to the configuration of the equipment (adding/removing/replacing modules), changes in the intended use of the equipment, maintenance and repair activities, and software and firmware

updates. It is advisable to ensure that software and firmware versions are upgraded for all equipment within the same laboratory to avoid compatibility issues between different software and firmware versions.[23] When software and firmware are upgraded, the user should evaluate what the impact of these changes is on the accuracy and completeness of the system documentation (manuals) and SOPs. It should be noted that the impact of firmware changes is often underestimated. Revision can result in different menu options, different parameters being reported on the printouts generated by the equipment, differences in formulas used to calculate results, and in the case of modular equipment, compatibility issues with other modules or similar equipment. Often the change control program differentiates planned and unplanned changes. Examples of planned changes are relocations, changes in intended use, software upgrades, and preventative maintenance activities, while unplanned changes include repairs and firmware upgrades, which are often installed during the preventative maintenance of the equipment with the knowledge of the end-user.

F. Maintenance

(Preventative) Maintenance activities for equipment are mentioned in both the US regulations (21CFR211.67[24]) and the EU regulations (both in Annex 15[7] and Annex 18[4]). In addition, the USP[21] requires that a preventative maintenance program should be implemented.

The minimum requirements, as described by the regulators, are establishing and following procedures that include:

(a) assigning responsibility for cleaning and maintenance;[4,24]
(b) maintenance, cleaning, and where appropriated sanitization schedules;[4,24]
(c) sufficient details of methods, equipment, and materials used in maintaining and cleaning operations;[4,24]
(d) protection and inspection of clean equipment against contamination prior to use.[4,24]

The EU regulators[4] have one additional requirement, namely to establish the maximum time that may elapse between the completion of processing and equipment cleaning, when appropriate. In addition to these regulatory requirements, (preventative) maintenance activities are performed for business reasons. They are a cost-effective approach implemented when equipment components require frequent replacement due to normal wear and tear.

G. Risk Management

The European regulators described that a risk assessment approach should be used to determine the scope and extent of validation. The

likely impact of a change of facilities, systems, and equipment on the product should be evaluated to determine the need for, and the extent of, re-qualification and re-validation[7], and if a "computerized system replaces a manual operation, consideration should be given to the risk of losing aspects of the previous system, which could result from reducing the involvement of operators."[9]

Prior to the FDA's *cGMPs for the Twenty-First Century: A Risk-Based Approach*,[25] the risk concept was only described in the Agency's guidance document on software validation.[12] This guidance recommends an integration of software life cycle management and risk management activities. Based on the intended use and the safety risk associated with the software to be developed, the software developer should determine the specific approach, the combination of techniques to be used, and the level of effort to be applied.

The pharmaceutical industry applies the similar risk-based approach in their equipment qualification programs, enabling them to make cost-effective decisions on extend and rigor of their qualification activities based on the criticality of the equipment. Multiple guidance documents describing how to incorporate risk principles into equipment qualification programs, made available by ISPE,[26] ICH Q9,[27] and ISO-14971, provide more background information on the general principles of risk management.

H. Documentation

Regulators in the US and EU require that qualification and validation activities are documented and that the documentation is properly maintained. The decision to approve and release a process for routine manufacturing is based on the review of all validation documentation, including data from the equipment qualification.[1] The focus of this section will be on the qualification documentation in the strictest sense of the word; the required SOPs. Logbooks and maintenance records will not be addressed. Annexes 15 and 18 of the *EU Guide to Good Manufacturing Practices*[4,7] provide a more detailed outline on which type of qualification and validation documentation is required, while the US Guideline[1] provides more detail on the content of the protocol.

The types of documents identified are given below.

1. Validation/Qualification Master Plans

The key elements of the validation program are summarized in a Validation or Qualification Master Plan (VMP or QMP, respectively). This high-level document answers at a minimum the following questions:[7]

- Which corporate validation/qualification policies apply?
- How are the validation/qualification activities organized?

- What is the scope of the project: which processes, facilities, systems, and equipment require validation, or qualification?
- How and where are the results of the qualification and validation activities documented?
- How will changes be controlled?
- Which documents are already available and where are they located?
- What are the roles and responsibilities for each group (e.g. owner, key user, quality assurance, metrology group, and external service providers) involved in the qualification/validation activities?

Investing time to develop a master plan enables companies to efficiently allocate resources and communicate timelines involved with the qualification/validation activities to internal and external customers. It should be noted that although equipment manufacturers, consultants, and other service providers can assist in the qualification/validation activities, the end responsibility for equipment qualification lies with the end-user. The end-user establishes the extend of qualification needed to demonstrate the suitability of the equipment, based on the intended use of the equipment, and identifies who will conduct the required test (in-house, consultants, manufacturer/supplier). The end-user's responsibility has been illustrated by Furman et al.:[23]

> . . . If the company who made our scientific apparatus could be sued because we cannot control the quality of the data it gave us, we would soon be forced to build our own liquid chromatographs and write our own computer programs to run them. The manufacturers would quickly abandon us for greener, less litigious pasture . . .

2. Validation/Qualification Protocol

A validation/qualification protocol is a written plan that states how validation/qualification activities will be conducted. The protocol should be reviewed and approved as outlined in the VMP/QMP.[1,4,7] At a minimum, the protocol must specify:

- Type of activities conducted (retrospective, concurrent, or prospective[4]). It should be noted that the *EU Guide to Good Manufacturing Practices* has identified these three types of validation activities, while the FDA's *Guideline on General Principles of Process Validation*[1] only defines prospective and retrospective activities. It is also advisable to document why the activities are conducted. Possible examples are new installation, relocation, after repair, change in intended use, and changes in acceptance criteria.
- The scope of the activities to be performed: functions/parameters tested and unique identification of the system. To achieve a cost-effective qualification/validation program, only test the functions

and parameters in a range that the end-user is intending to use. The functions that are available but which are currently (and in the nearby future) not used can be placed out-of-scope. For modular systems, it is advisable to record the name of the manufacturer (or supplier), model number, serial number, other identifying numbers, and soft- and firmware names and versions (when applicable) for each component of the system.

- Critical steps and decision points on what constitutes acceptance test results ("acceptance criteria"). All acceptance criteria must be met during testing. If a test shows that the equipment does not perform within its specification, a root cause analysis should be performed, corrective action should be implemented, and additional tests should be performed to verify that the equipment can perform within the predetermined specifications.[1,4,7]
- Actions required to be taken in accordance with corporate procedures in the event a test does not meet the predetermined acceptance criteria.
- The tests to be conducted, including test parameters and the data to be collected. The purpose for which each data are collected must be clear, the data must reflect facts, and the data must be collected carefully and accurately.[1] It should also be identified who is responsible for execution (key user, external service provider (ESP), equipment manufacturer, . . .) and reviewing each test and the data generated during testing.
- The number of test runs required to demonstrate reproducibility and to assure reliable and meaningful results.[1,4] This number can be determined based on statistical analysis of historical data, ICH guidelines on method validation (e.g. accuracy testing) and/or technical requirements defined in the applicable pharmacopoeias (USP, British Pharmacopoeia (BP), European Pharmacopoeia (EP), etc.)
- The required test equipment (e.g. caliper, tachometer, flow meters, etc.) and test solutions/standards for each test and documented evidence (e.g. calibration certificates) of the suitability of materials and the performance and reliability of equipment and systems.[1] The calibration status of each test equipment should be verified prior to execution of the each test.
- Worst-case conditions, which are defined by the FDA as "a set of conditions encompassing upper and lower processing limits and circumstances, including those within SOPs, which pose the greatest chance of process or product failure when compared to ideal conditions."[1]

It is important to realize that each test is written in such a way that it is able to detect potential failures, not just demonstrate that procedural requirements have been met.

3. Validation/Qualification Report

Annexes 15 and 18 of the *EU Guide to Good Manufacturing Practices*[4,7] define the purpose of the report that cross-references the protocol as follows:

- to summarize the obtained results,
- to comment on any deviations observed,
- to draw the necessary conclusions,
- to recommend changes to correct deficiencies,
- to document and justify any variations from the protocol, and
- to provide a formal release statement.

IV. DISCUSSION: CASE STUDY HPLC

In this section, a closer look will be taken at each of the individual modules of a high performance liquid chromatograph (HPLC) and the parameters that can affect the accuracy and precision of the analytical results will be discussed. Where available, commonly accepted tolerances for each parameter will be provided.

A. Detector

1. Wavelength Accuracy

Wavelength accuracy is important for a correct interpretation of data, for accuracy of the obtained results, and when conducting method transfers. It is determined by comparing a measured absorbance with the absorbance maxima of a certified standard (e.g. Holmium oxide filter). The European Pharmacopoeia (EP 2.2.25) describes the permitted tolerances for absorption spectrophotometry in the ultraviolet (UV) and visible range (± 1 and ± 3 nm, respectively), while the USP[16] indicates that recalibration is required when the observed wavelength differs more than 3 nm from the certified standard value.

2. Baseline Drift and Signal-to-Noise Ratio

Both baseline noise and signal-to-noise ratio are important in the determination of the quantitation and detection limit. Typically, accepted signal-to-noise ratios are 2:1 and 3:1 (detection limit) and 10:1 (quantitation limit).[13,14] ICHQ2B[7] describes several approaches to determine the detection limit based on:

- visual evaluation,
- signal-to-noise ratio,
- standard deviation of the blank, and
- calibration curve.

3. Linearity

A linear relationship between the concentration of the analyte and the output of the detector is expected. Detector linearity is important for a correct interpretation of data and the accuracy of the obtained results. The detector must have a broad linear dynamic range.[16] It is determined by injecting a series of certified and traceable standards (e.g. caffeine) covering the range of the equipment. ICH[13,14] recommends that a minimum of five concentrations is used to determine the linearity. Typically, acceptance values for the correlation coefficient are >0.999.

B. Solvent Delivery Systems (Pump)

1. Inert Materials

The components of a pump systems used in quantitative analysis should be constructed of materials inert to corrosive elements in the mobile phase.[16]

2. Gradient: Accuracy and Precision

Gradient fluctuation can affect retention times, peak shape, and integration results (e.g. differences in observed peak areas). The accuracy is determined through introduction of a tracer through one of the solvent channels and measuring its concentration in the mobile phase, while the precision is determined by performing several gradient measurements over time. Typical tolerances dictate an average recovery accuracy of between 95 and 105%.

3. Flow Rate: Accuracy and Precision

Flow rate accuracy and precision are important for a correct interpretation of data, for the accuracy of the obtained results, and when conducting method transfers. Flow rate fluctuation can affect retention times, peak shape, and integration results (e.g. differences in observed peak areas). The accuracy is determined by measuring a volume of mobile phase delivered over a predetermined period of time (e.g. flow meter), while the precision is determined by performing several flow rate accuracy measurements over time. Typical tolerances are ± 3–5% (accuracy) and %RSD (retention times) $<0.5\%$ (precision).

4. Flow Rate: Stability

Both the USP[16] and EP (2.2.29 Liquid Chromatography) indicate that an HPLC must be capable of delivering the mobile phase at a constant flow rate with minimum fluctuations over "extended periods" of time. It is an acceptable practice to run these tests for 16 h demonstrating that the instrument is suitable for overnight analysis.

C. Column Compartment

1. Temperature Accuracy and Precision

Fluctuations in column temperatures can affect detector response and the separation processes resulting in retention time variations. The accuracy is determined by measuring the actual column temperature and comparing it to the temperature set-point of the instrument. The temperature precision is obtained by performing several temperature accuracy measurements over a period of time. Typical tolerances are ±1–2°C.

2. Temperature Stability

Temperature stability studies are conducted to demonstrate that the instrument is capable of maintaining a temperature set-point over an extended period of time and therefore is suitable for running overnight analysis. It is an acceptable practice to run these tests for 16 h demonstrating that the instrument is suitable for overnight analysis.

D. Injector/Autosampler

1. Injection Volume Accuracy and Precision

During most analysis the analyst is more interested in volume precision than volume accuracy. Volume precision is a important factor in the instrument's repeatability and is determined by injecting a standard a minimum of six times[13,14] and calculating the RSD value. Commonly used tolerances are %RSD < 1.

2. Injection Volume Linearity

Since most analytical methods do no require variable volumes to be injected, volume linearity is of lesser importance than volume precision.

3. Temperature Accuracy and Precision

Cf. Supra – Section IV.C.

4. Correct Vial Processing

It is advisable to investigate how the HPLC system reacts when a vial that should be picked up and injected into the system is not present. The system should at a minimum generate an error message and the user should be prompted to take corrective action.

5. Carry-Over Testing

Usually a method-specific criteria, but can be tested in initial equipment qualification. A blank sample is injected after a high-concentration sample, it is expected that less than 0.05% of the analyte of interest can be found in the test results of the blank sample.

E. Computer/Software Validation/Firmware

US and EU regulators have in common that they both require that computers and software are used to control laboratory instruments and/or process data generated by these instruments, but they have a different approach. In Europe, Annex 11 focuses on computerized *systems* that "replaces a manual operation,"[9] while in the US the FDA focuses in the 21CFR11 regulations on the *electronic records* that are "created, modified, maintained, archived, retrieved, or distributed by a computer system."[10] As stated earlier, an authoritative guidance on software validation already exist[12] (cf. Supra – Section II.G), therefore this will not be discussed in detail in this chapter. The end-user should have documentation available describing the applicability and restrictions of the commercially available software package. This can be achieved by retaining the user manuals and additional information provided by the original equipment manufacturer (OEM) of the software packages. The end-user should also ensure that the software packages "are to be operated as intended and within the limitations specified in the manufacturer's manuals and documentation."[23] Unlike commercially available off-the-shelf software, the user is responsible for the validation of any software applications that are developed in-house in accordance with the available authoritative guide.[20] The validation of the software packages used to control the equipment and acquire/process data should be an integral part of the equipment qualification program. Too often, requirements associated with software validation and 21CFR11 and Annex 11, respectively, are treated as separate entities. For example, verification of security and audit trails requirements can be incorporated into the equipment qualification program, thereby demonstrating compliance with both software and 21CFR11 and Annex 11 requirements. Firmware is considered to be an integral part of the equipment. Therefore, no further validation activities are required, especially because the end-user often cannot alter the firmware and the equipment will not operate as expected when the correct firmware is not installed. However, this does not mean no attention should be paid to firmware during equipment qualification. It is advisable to record the different firmware version of each HPLC module. For example, apparent identical and interchangeable modules (e.g. detectors) could no longer function as expected after they were introduced in a similar system, following corporate change control procedures, due to conflicting firmware versions.

V. SUMMARY AND CONCLUSIONS

Eliminating competing interpretations of requirements and confusion about what exactly is required to achieve compliance with increasing

regulatory expectations in the pharmaceutical industry's equipment qualification programs can only be attained by pursuing a higher level of consistency in the terminology used and services provided by external service providers and authoritative guidance documents that clearly describe the regulatory expectations.

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10 PHARMACEUTICAL DEVELOPMENT: FROM PRE-CLINICAL TO POST APPROVAL

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ABSTRACT

High performance liquid chromatography (HPLC) is a flexible tool that is utilized to generate the majority of analytical data that is required during the drug development process. HPLC methods can be modified to meet most of the challenges demanded from the scientific, business, and regulatory driving forces during the entire drug development process, from the discovery of the therapeutic compound, to the final commercialization of the product. This chapter provides an overview, of the changing requirements of the drug development process and how HPLC methods evolve during this process.

HPLC Method Development for Pharmaceuticals
S. Ahuja and H. Rasmussen, editors.

I. INTRODUCTION

High performance liquid chromatography (HPLC) is a very powerful and flexible tool that forms the analytical backbone for the entire drug development process, from discovery to the final release of commercial product.[1] One of the key advantages of HPLC is that this technique is extremely flexible and can be adapted to fulfill the analytical needs of each stage in the drug development process. The drug development process first begins with unknown and uncharacterized compounds, or mixtures of compounds, that have not been fully characterized. The methodologies that are utilized at this stage to first characterize the substance, must then be employed later in the development phase, in a more regulated good manufacturing practices (GMP) environment. In this role the HPLC technique will still solve analytical problems, but the added burden of doing so in highly regulated and controlled manner, creates new hurdles for the development laboratory. Finally, the analytical technique will need to be transferred to the quality control laboratory to ensure the purity, and efficacy of the commercial product. This technique will need to be simple, rugged, and robust and can be used in the quality control laboratory with minimal training. The method must also be "QC" friendly, in that it must be cheap, fast, and should not generate errors, since these will lead to costly manufacturing investigations.

This chapter will serve to describe the evolutionary process of how HPLC methods are developed and utilized at each stage of the drug development process to fulfill the scientific, regulatory, and business needs of a pharmaceutical company. The key factors of such a method will be discussed, as well as how they evolve across the entire process.

There are three driving factors that determine the type of analytical techniques which are needed at each stage of the drug development process. These three factors are the regulatory requirements, the scientific requirements, and finally the business requirements.

1. Scientific Requirements

The cutting edge of chemistry, biology, and physics is utilized to synthesize, select, and develop new pharmaceutical formulations. Throughout the development process it is critical to learn as much about the compound under study as quickly as possible. In this manner a therapeutic indication for the compound can be determined as well as any potential adverse indications that may lead to the demise of the compound. The experiments that are conducted during the discovery and the pre-clincial phases will form the scientific foundation that will provide the critical knowledge base for the compound. This "critical mass" of knowledge will be utilized later in development and clinical phases to ensure that a safe and effective product is developed.

2. Regulatory Requirements

Worldwide, the pharmaceutical industry is one of the most heavily regulated industries. No product can be marketed or sold without the permission of the relevant regulatory authority. As a result one of the key drivers in bringing a pharmaceutical substance to market is complying with all of the relevant regulatory requirements. These requirements generally call for the generation of a dossier, based on a series of clinical and analytical studies which will demonstrate the safety and efficacy of the product. In addition the pharmaceutical organization itself will have to demonstrate that it has the required quality systems in place that will allow for the development and continued production of such products. These quality systems will tend to slow down the development process, and cost additional resources, but the cost of not implementing the required quality system can be much greater.

3. Business Requirements

The end result of any pharmaceutical venture must be the generation of profit. In order to realize such a profit, the product must be produced, tested, and released as inexpensively as possible. This will drive the selection of synthetic processes, and analytical testing methodologies utilized for the final product. The phrase "time is money" can be applied here, since there is a finite lifetime for a patented product on the market. This will strongly encourage fast processes and analytical methodologies.

A. General Trends for an HPLC Method During the Drug Development Process

During the drug development process the HPLC methodology utilized will change as the driving forces of the scientific, business, and regulatory forces require. This continual change is illustrated in Figure 1.

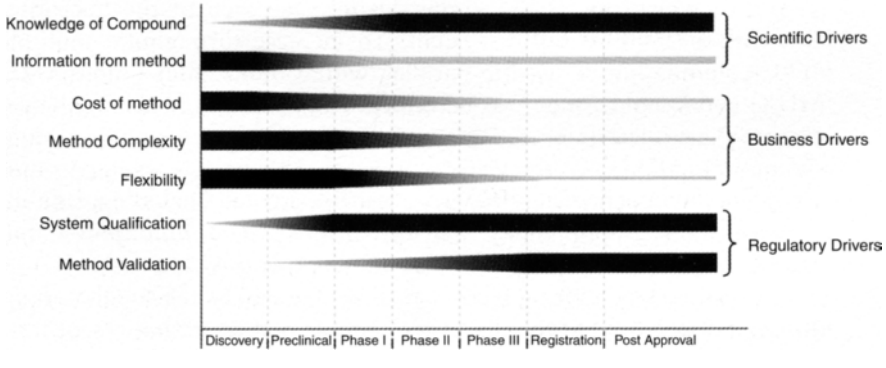

FIGURE 1 Driving factors during the drug delivery process.

During discovery and early development, there will be little knowledge about the compound under study, so the scientific requirements will demand advanced methodologies that will collect as much information as possible. This will result in very expensive and complex methods. At this point there will be few regulatory requirements, so test methods can be quite flexible. During the clinical and development phases, the bulk of the scientific information about the compound will have been generated, so simpler and cheaper methods will be utilized. Additional regulatory burden will be required at this stage, such as method validation and system qualification. This trend will continue to the point of method transfer, where simple, fast, and cheap methodologies will be utilized to release the finished product under a highly regulated environment.

II. THE ROLE OF HPLC IN DRUG DISCOVERY

Modern trends in drug discovery utilize a high throughput screening (HTS) approach to find new active pharmaceutical ingredients. The scope of modern HTS approaches requires fully automated systems to screen the huge volume of compounds generated by this methodology.[2] An HPLC method that is utilized as part of such a screening methodology must be rapid, and highly automated. Current trends in this methodology utilize fast HPLC methods coupled to a selective detector(s) such as a mass spectrometer.[3] Quite often orthogonal separation, and/or orthogonal detection methodologies are used either in series or in parallel.[4]

The HTS approach generates a huge number of candidate substances in a microtiter plate format. These samples must then be analyzed for a number of key factors that will determine if they will be likely candidates for further development. The end result is that the HTS laboratory needs to process and screen many thousands of samples, each of which is an unknown mixture, and each of which is present at relatively low concentration levels. This is typically accomplished utilizing a robotic sampling apparatus, which injects the samples from each well of the microtiter plate into a standard HPLC system. To increase throughput, multiple HPLC columns can be used in parallel, with a multichannel multiplexer (MUX) connected to the mass detector.[5] The purpose of such analytical screening is to determine the adsorption, distribution, metabolism, and elimination (ADME) of the lead compounds. During this testing a number of key physicochemical properties are investigated, including, solubility, permeability, lipophilicity, and cytochrome p450 inhibition. A full discussion of these assays are beyond the scope of this chapter.

One of the key limiting factors at this stage will be the small amount of material available to the discovery chemist. An entire battery of tests must be conducted, on a very limited amount of material. The tests that are conducted must be carefully planned, so that the information that is

required is gathered, and with the consumptions of as little sample as possible. One example of a strategy to conduct such a battery of tests during lead selection is presented by Balbach.[6]

In each of these cases the HPLC technique is typically not the key source of information, the HPLC separation is coupled to a second more specific detection methodology such as mass spectrometry (MS). Typically a very simple, and fast reverse phase HPLC method is utilized. Such methods typically consist of a rapid linear gradient of dilute (0.1%) formic acid as an aqueous component, and acetonitrile or methanol as the organic component. The column is typically a short (2–3 cm) C-4, C-8, or C-18 with particle sizes of 2–5 μm. A fast linear gradient is then run at >3 ml/min over about 3 min from 0% to 90% organic modifier, providing partial separations.

The use of a standardized fast LC method is necessary for two reasons. First, the huge volume of samples requires that a very rapid separation technique be utilized. Even if the individual compounds are not separated, the specificity of current mass detectors can easily identify the components that are not resolved by the HPLC system. Second, the huge diversity of compounds synthesized makes it impossible to develop a single HPLC method that will resolve all potential mixtures encountered during the HTS process.

During such a mass screening approach the HPLC method essentially becomes an elegant sample introduction technique, to a more specific spectroscopic technique. Any separation that does occur will be useful, since it will simplify the spectroscopic analysis, but chromatographic resolution is not a requirement.

The goal of a method development chemist who seeks to develop such an HTS method is to develop as fast a method as possible, that can introduce as many samples as possible to the selective detector. At most a chemist will develop a few different HPLC methods based on the class of compound being studied, since tailoring an HPLC method to each compound under investigation is impractical.

The key decision is then which detection methodology is to be used. A number of selective detectors can be used individually or in parallel, to generate orthogonal data from each sample. One general strategy is to utilize a less selective, more universal detector, such as a UV or evaporative light scattering detector (ELSD) in parallel with a more selective detector such as a mass spectrometer. This approach ensures that all compounds that have been separated are detected, even if they are not ionized by the ESI source in the mass detector.

Finally new technologies such as ultra performance liquid chromatography (UPLC)[7,8] are becoming available as a tool to be utilized in the HTS process, and will be able to improve the throughput of current automated approaches, due to the decreased runtimes observed on such systems.

A. Driving Factors for HPLC Method Development in Drug Discovery

During the discovery phase the key driving factor is the scientific requirements. The HPLC methods that are utilized at this stage generate information about the compounds that are being screened. During the discovery phase, as much knowledge about the compounds must be collected as quickly as possible. The goal is to eliminate candidates that will fail at later (and more expensive) clinical stages of development, as early as possible. The analytical tools at this stage represent the cutting edge of analytical science. As much information is collected, as rapidly as possible about the compounds being screened, so that the key decisions can be made. Advanced techniques such as HPLC-MS and HPLC-NMR will be employed to identify, quantify, and characterize the compounds being screened. The advanced techniques utilized also require that highly skilled individuals be part of the drug discovery process. Highly trained (doctorate level) and experienced scientists will be responsible for all of the analytical work conducted at this stage, to ensure that the best science that can be done is conducted. The end goal is to make accurate and rapid decisions about as many compounds as possible, so that only the most likely candidates are promoted to development.

Since no humans will be exposed to these compounds in a clinical setting, there are no regulatory requirements for these methods. As a result, the typically cumbersome requirements of cGMP work, such as change control, method validation, and system qualification, are not required (although some validation may be needed from a business perspective). This means that HPLC methods at this stage can be much more flexible and can be changed when deemed scientifically necessary.

The analytical tools are used to make the critical decisions to decide which compounds should be promoted to full development status. Because of this it is absolutely critical from a business perspective that any compound that would not be safe or effective be screened out during the discovery phase. If a candidate compound, which would not be safe or effective, is not screened out during the discovery phase and makes it to the development phase, then it will likely fail one of the much more expensive clinical trials. This not only hurts the company in terms of the cost of the failed clinical study, and other development costs, but it also wastes some of the company's pipeline resources.

The discovery phase is a collaboration of the scientific and business functions of a pharmaceutical company. The best science that is available must be brought to bear in order to screen as many potential drug candidates as possible. The scientific tools must be able to screen out as many compounds as possible that will fail later stages. This is the most cost effective and safest approach to drug discovery/development.

Since none of the compounds in this development stage will be introduced to people, there are no regulatory requirements for this stage. All that matters is to find therapeutic compounds that will be safe and effective in later studies in both animal and human clinical studies. The role of HPLC in this process is to serve as a rapid separation tool for more advanced and complex analytical methodologies, which will provide the lion share of the information about the compounds being screened.

III. THE ROLE OF HPLC IN PRE-CLINICAL DEVELOPMENT

Once a compound has been promoted as a development candidate, the most immediate task will be to develop a stability indicating HPLC method for the compound of interest. This HPLC method is needed to fulfill the many tasks of pre-clinical development. HPLC will rapidly become the methodology of choice for the purity analysis and assay of drug substance prior to the initiation of a clinical study. All of the pre-clinical work is moving toward one goal, the generation of the investigational new drug application (IND) and the completion of the studies that are needed to move the compound into the clinical phase.

The first critical task in pre-clinical development, that the analytical development laboratory will undertake, is the support of the scale up synthetic process. It is likely that until this point the synthetic methods have been on the gram scale. The choice of synthetic route will be guided by a stability indicating method that will be able to resolve all of the synthetic impurities present in the product. Various synthetic processes will be evaluated utilizing the stability indicating HPLC purity method. If external vendors are being utilized to synthesize the material, then the data from this HPLC purity method will be used to rank the quality of the product from each external laboratory.

This method will then be used to release the initial batches of active pharmaceutical ingredient (API) for use in *in-vitro* and animal toxicological studies, which is critical in determining the safety of the product. It should be clear why a purity method at this point would be critical. If the HPLC method did not resolve an impurity, and a batch containing such an unresolved toxic impurity was released to a toxicological study, the study may incorrectly indicate that the candidate compound was unsafe. This could lead to the erroneous termination of the new compound, which may have gone on to be a safe and effective drug substance.

Other analytical HPLC work will also be needed to support toxicological studies. Our stability indicating method will be needed to conduct additional solubility studies. Quite often toxicological studies will call for the use of high concentration solutions. This will require additional solubility studies, potentially at different pH ranges.

Also at this stage initial stability studies will be conducted. Both open petri and sealed container studies on the raw material, as well as solutions, at various concentrations will be needed. This will not only indicate the stability of the material, under different conditions, but it will also provide an idea of the actual degradation products that will be observed under "normal" conditions. It will also demonstrate how stable the compound is in various solutions at different pH and concentration levels. Different container closures of the material are also needed at this point to determine the optimal packaging for the API. In each of these cases, the bulk of the work will be conducted utilizing a stability indicating HPLC assay purity method. HPLC-MS will be an invaluable tool during this initial experimental work, to ensure that all degradation products are being detected quantitated, and identified.[9] The method itself may need to be modified as these studies are conducted and new degradants are detected.

Finally, the chiral purity of the material may need to be characterized. This can be accomplished with a secondary method for chiral purity, or the primary purity method could be developed to resolve chiral compounds. This chiral method will be utilized to both evaluate the efficiency of enantiomerically specific synthetic processes, and to evaluate chiral purity over time.[10]

At this stage in development there is a critical need for a method that will identify and quantify all of the impurities in a drug substance. The first course of action will be to develop a stability indicating method that can be used to fulfill all of the tasks listed above. The question is where to start in developing such a method?

The first place to begin a method development is the chemical literature. A librarian once told me "Fifty hours in the lab will save one in the library". These are truly wise words, and can save the scientist many hours of fruitless trial and error. Quite often a model compound that has similar chemical functionally and structure has already been fully characterized by other groups, and the methods are available in the literature. If a method in a publication can be modified to provide an effective separation many days of development time can be saved.

If the literature provides no help, then a more methodical method development strategy must be employed. The exact method development strategies are discussed elsewhere.[11] One approach is to utilize orthogonal methods that utilize different separation mechanisms, so that the chance of coincidental overlap of impurity peaks with each other or the primary component is less likely.[12] This is an excellent approach that can be used to resolve all of the impurities in a drug substance, but requires that the laboratory have sufficient resources to run numerous assay methods for each sample tested. Another strategy is to rely on LC-MS to resolve overlapping peaks, which can then be separated through

method development strategies. Throughout the pre-clinical development phase LC-MS should be routinely employed to ensure that all of the impurities and degradants are resolved.

Once an initial method(s) has been developed, it must be challenged with samples that are impure to see if the method(s) are capable of resolving the impurities. This leads to the question, where do we get an impure sample? One source of impurities will be the drug substance itself, there may be a number of impurities that can be separated and identified by MS. This may be able to shed some additional information about the process impurities in the current synthetic process. Additionally, it may be possible to obtain crude (unpurified) synthetic material, or intermediate synthetic samples, that will have these process impurities in larger amounts. These impurities can then be used to tweak the method, so that all impurities are effectively resolved. Once process impurities are accounted for, a forced degradation study is required, which will generate impurities, that must be resolved by the HPLC method. A number of examples of forced degradation studies are presented in the literature.[13] Ideally this process will yield a method that provides mass balance between the undegraded drug substance, and the remaining drug substance and all degradation products.[14] The use of HPLC-MS to ensure that all degradants are identified is critical. The determination of mass balance is further complicated because it is likely that the UV response of the degradation products will differ from the response of the parent drug substance. The use of a universal detector such as an ELSD or a nitrogen detector can be very useful for this task.

A. Driving Factors for HPLC Method Development in Pre-Clinical Development

It should be evident from the above discussion that the critical role of HPLC during pre-clinical development is to serve as a stability indicating purity method. A method which is able to identify and accurately quantitate the active component, even in the presence of degradants and impurities, and be able to further quantify the level of degradation is critical. This method is needed to demonstrate that all material that will be utilized in animal and other pre-clinical studies is pure. This results in a strong scientific driving force, which requires that we know what material we are dosing the animals with, in order to interpret any adverse events. Otherwise, if we dosed animals with an impure compound, and an adverse event was observed, we would not be able to determine if this toxic effect was due to the main component, or the impurity that was dosed along with it.

The analytical support that will be provided during the synthetic scale up process is also of critical importance. The synthetic chemistry group will need to have an analytical tool that will allow them to rank

their synthetic methods on a basis of yield and purity of product that is produced. If this tool does not provide accurate results, then the synthetic group is effectively operating blind. The feedback provided by an accurate purity method is critical. The method should initially be able to at least resolve the API from all of the synthetic precursors, and any undesirable degradation products, and products of side reactions. Additionally, it should be able to resolve any impurities that may be present in the starting materials themselves.

The regulatory risk at this stage for the scale up analytics is fairly low, but the business drivers are huge. An incorrect purity method that misleads the synthetic group towards a synthetic procedure that creates an impure product will at best require a considerable re-working of the process when the impurity is eventually discovered.

Some organizations will choose to source an active ingredient from an external vendor. In this case, the business criticality of the pre-clinical HPLC method is even greater. This tool will be one of the major metrics used to evaluate the product that will be purchased from an external vendor. A purity HPLC method that is capable of identifying and quantitating all impurities will be invaluable in determining which vendor will be able to supply the purest product. One note of caution is that many vendors of raw material may be happy to provide their own purity method; the reader should be cautioned by this seemingly generous gesture of a complete package, of raw material and complementary analytical methods. The free method that is provided, may not be able to resolve all (any) of the impurities in the vendors product, creating a false impression of a high purity product.

Another key deliverables that a pre-clinical HPLC method will provide is support for the initial toxicology studies. These pre-clinical studies will be the first studies that need to comply with a regulatory guideline. These initial HPLC methods will be used to release material for toxicology and initial animal studies, and to fully characterize both the test article (API), and test solutions. As a result all methods will have to comply with the relevant Good Laboratory Practice (GLP) guidelines. The GLP guidelines state that:[15]

> *The Identity, strength purity, and composition or other characteristics which will appropriately define the test or control article shall be determined for each batch and shall be documented before the imitation of the study.*

This requires that a stability indicating method be used to qualify and release each batch of material prior to the initiation of any study. The current GLP regulations also require the running of a stability study of the test solutions, again, requiring a stability indicating method. There

will of course be a business driving force present here. An incorrect analytical result could jeopardize a costly toxicology study, and result in inaccurate results. This could not only require a repeat study, but if the error is not caught, it could result in the incorrect assumption about the safety of the compound, resulting in the termination of a product that may in fact be a safe and effective therapeutic compound.

Also at this stage the regulatory requirements require that some method validation be conducted. This will necessitate the use of traceable methods (often documented in a notebook) which are version controlled. Each method will then need to be validated to a level that is appropriate for this level of development.[16] Typically linearity, specificity, and reproducibility are studied at this stage of development. All of the degradants may not be fully known or characterized, so the purity will usually be expressed as a percent of total peak area of the parent compound. The equipment that these methods are conducted on must also be qualified and calibrated.

This added regulatory burden will result in a reduction in flexibility, and will require more paperwork for each sample. For example, if a change needs to be made to a method, to resolve a new impurity, the new method must be developed and fully documented as a new method. An abbreviated validation study should then be conducted on samples that contain all of the impurities, to demonstrate that all known impurities can be resolved. Additionally, a linearity study is needed to show that the main component is within the linear range of the detector. Finally, the limit of quantitation of the new impurity should be measured and documented.

One of the key business requirements during the pre-clinical phase is pilot plant scale up support. The HPLC method at this time will be used to evaluate samples of drug substance produced under different synthetic schemes. The synthetic organic chemists will seek to develop the simplest and cheapest synthetic method, which produces the "cleanest" drug substance. It is absolutely critical at this point that an HPLC method be available that can identify and resolve all synthetic impurities that are present in these samples. This analytical feedback is critical, because this information will be used to select a synthetic process that will produce all future batches for clinical and technical use in formulation development. If an unresolved impurity is not identified it could jeopardize these more expensive future studies, and result in a development dead end, if a new (cleaner) synthetic process had to be utilized during clinical development.

IV. THE ROLE OF HPLC IN CLINICAL DEVELOPMENT

The goal of the clinical development process is the successful submittal and approval of a new drug application (NDA), which will allow a pharmaceutical company to market and sell a drug product. During

this phase the analytical development group completes a number of tasks that are critical to a successful NDA submission. These tasks include the development of a successful formulation, release, and support of batches for clinical study, the analysis of stability of both the raw materials and finished drug product, and finally the transfer of the analytical methods to external laboratories such as quality control, or contract laboratories.

The first step in the clinical phase is the development of a dosage unit that is suitable for human consumption. For a typical immediate release formulation, the initial dosage unit is often in the form of a gelatin capsule that is filled with a mixture of the active ingredient and an additional filler material such as lactose.[17] These capsule formulations will often be used for early clinical studies, even if the final market image will be in the form of a tablet. Initial HPLC methods that were used for the API in pre-clinical phases will often be able to track the assay and purity in these early clinical formulations. The goal of this simplistic formulation is to get the API into humans as fast as possible to determine if the API is bioavailable. If these initial studies are successful, then a second phase of formulation development will likely be implemented, which will seek to incorporate the active ingredient into a tablet form.

The early clinical formulations will be placed on stability at recommended, accelerated stability and often open petri conditions, to see how stable the formulation is under these conditions. Two types of degradation products are likely, the first results from the degradation of the API from environmental factors (heat, moisture, light, oxygen). If the preclinical method was successfully developed, then the initial method from early development should effect a separation of the active component from these impurities. The second class of impurities is degradants that result from the interaction of the API with the excipients in the formulation. These impurities may or may not be resolved from the active ingredient and/or other degradation products. In order to determine if the method is still suitable (stability indicating) additional peak purity measurements should be made using LC-MS on these accelerated samples. Mass balance should still be observed under these accelerated conditions. These initial formulations may prompt additional method development, in order to resolve all of the degradants, from the main peak, and any excipient peaks. These optimized methods will then be utilized to monitor the stability of the formulation, to see if the API will be stable enough to be used in a clinical study. If the level of drug plummets 20% in three months at recommended storage conditions, then it is clear that either the excipients, or packaging is not suitable for the compound. Additional stability studies will be needed with different excipients, or packaging changes (desiccant, Al–Al blisters). If the stability of the API is still not suitable, then this is a strong driving force to kill the compound, before any costly clinical studies are executed.

At this formulation development phase dissolution testing will become very critical, for formulation development, and stability studies for the drug product. In many cases, especially for multicomponent formulations, HPLC will be utilized as a tool for analyzing dissolution samples. Although the stability indicating assay purity method can be utilized, a much faster method that just resolves and assays the active ingredients is desirable in order to speed the analysis of dissolution samples.

Once enough stability data from a technical batch is collected, which indicate that the formulation is stable under relevant storage conditions, the decision to begin a clinical study can be made. The R&D analytical laboratory will now function as the release laboratory for the clinical material that will be used in the study. The HPLC assay purity method will be a key tool in the release of this material, since it will show that the correct amount of API is present, and that the level of potentially toxic impurities are not above levels that would be considered safe. It is critical that at this point that the method is stability indicating and that it is validated to a level that is ". . . suitable for their intended purpose".[18,19] This requirement is a bit vague and its exact meaning is subject to interpretation. If one looks at the purpose of an early clinical trial it becomes evident that these early clinical studies are designed to deliver a specific dose of therapeutic compound, in order to determine a blood level profile in healthy individuals. One would expect that such a method would be able to accurately assay the level of therapeutic API with the exclusion of other impurities and degradation compounds. This would require that the accuracy, repeatability, linearity/range, and specificity be studied at this point in development. The limit of quantitation (LOQ) of the individual impurities should also be considered. Validation parameters such as robustness and intermediate precision are not as critical at this point since only one analytical group, and most likely, one individual chemist will perform all of the laboratory work to release early clinical batches at this point in development. At later clinical studies, as the product approaches the commercial stage, it will become necessary to validate that the method is robust and can be run by multiple chemists in different laboratories.[16]

Regardless of the logical justification of the level of validation of methods which ". . . is suitable for their intended purpose" there should be a company standard operating procedure (SOP) that specifies what validation experiments are required for each phase of development. This SOP should clearly define exactly what level of validation is required for each specific phase of clinical study.

Also at this stage, all systems used to generate data for the clinical study must be validated in compliance with 21 CFR part 11.[20]

One additional task that HPLC will play a critical role in is the cleaning validation of manufacturing equipment. A cleaning validation method that is both sensitive and specific for the compound that is being manufactured is required to ensure that the production equipment which comes into contact with the API is cleaned properly.

The analytical R&D department will also support the production activities that lead to a successful NDA. These include scaling up the process to a commercial level, validation of the production process, and transferring the method to an external QC laboratory. Once a formulation has shown promising clinical results, the formulation process will be locked, scaled up, validated, and a number of batches made for registration stability. Since all of the data from these activities will be presented to the relevant regulatory authorities, there is a large regulatory burden.

This stage will result in a large number of samples entering the laboratory, and an increase in the amount of regulatory work that is required. Methods at this point may be streamlined and/or optimized. Robotic sample preparation is often introduced and validated at this point. If multiple orthogonal methods were previously utilized, there will be an attempt to consolidate these methods into one method so that the number of HPLC sample runs can be minimized. One example is the analysis of chiral purity. If it can be shown that the starting material in the process is optically pure, and the chiral purity does not change on stability, then these tests may be dropped for the finished product. This will make the laboratory run more efficiently, and will facilitate the analysis of the large number of samples that will be generated in process validation and registration stability. Methods will also be simplified wherever possible, so that less skilled analysts will be able to run the analysis. This will culminate with the transfer of the method to the QC laboratory where efficiency and ease of operation are paramount. Following the method optimization the final methods will be validated completely in compliance with the ICH guidelines,[21,22] which will include all method validation parameters including robustness, and reproducibility. This will often be part of the method transfer, where the reproducibility will be run at the R&D laboratory, and QC laboratory simultaneously.[23]

A. Driving Factors for HPLC Method Development in the Clinical Development Phase

The Clinical Development process can be broken into two portions, early phase clinical (Phase I) and late phase clinical/registration (Phase II/III). In the early clinical studies the key is to determine as quickly as possible if the API is bioavailable at a safe therapeutic dose. This is a key turning point, if therapeutic levels can be obtained, then the drug may be a successful product. If at this stage the drug proves to be not bioavailable, then common sense would demand that the compound development be stopped, and resources be shifted to a more promising compound.

During this early phase the key scientific driving force is to ensure that an accurate dose of pure component is delivered in the dosage unit.

The HPLC method must be able to accurately accomplish this task, and if it has proven to be stability indicating, and mass balance has been observed, this requirement should be easily met. The key business driver is to get the API into man as fast as possible, so the key "go-nogo" decision can be made as rapidly as possible. The accurate stability indicating HPLC assay purity method makes this possible since this is the method that will be used to release the clinical batch. Any failures in the HPLC method to accurately meter the purity or potency of the dose could jeopardize the clinical study.

Finally, the regulatory requirements begin to play a much more prominent role, as soon as the formulation begins to be utilized in a clinical setting. The analytical methods must be validated at a level that is ". . . suitable for their intended purpose". This would mean that validation factors which demonstrate that the method measures an accurate amount of drug substance must be tested. These factors, accuracy, repeatability, linearity/range, and specificity, should be tested at this stage of development. All systems which generate data to support the clinical trial must be fully validated in accordance with 21CFR Part 11. This will include the analytical instrumentation, chromatographic data systems, and laboratory information management systems (LIMS). Any failure to comply with these regulatory requirements will expose the company to a large risk of a regulatory agency taking punitive action, which can have a dramatic impact on the core business of the company. This feedback between the business and regulatory requirements is becoming a critical factor to consider, due to recent instances of regulatory agencies imparting large fines, and consent decree agreements on a number of pharmaceutical companies.

In the late phase clinical/registration phase of development, there will be a high confidence in the product as to its efficacy and safety, due to successful early phase clinical results, which will allow for the promotion of a compound to these more extensive and therefore expensive studies. The key goals of this phase will be to generate a collection of data that will be used to demonstrate that the compound is safe and effective, in order to get approval to market the product. The later phase clinical results will demonstrate the level of efficacy and safety, and any potential side effects of the therapeutic compound. Again HPLC will be the methodology, which plays the major role in the release of drug product to these clinical studies. The regulatory requirements will play a major role during this period, since all of the data that is collected will be presented to the relevant regulatory agency.

Additional process validation studies will demonstrate that the formulation can be produced in a controlled and reproducible manner. A standard process validation methodology has the potential to generate huge numbers of samples, which must be tested by the HPLC method. There are two basic ways to deal with a deluge of samples, either to

"burn calories" or "work smarter". Given enough resource (head count, instruments) any number of samples can be tested quickly and accurately, but at a huge cost. The burning calories approach is costly, and is not favored by the business driver that requires an efficient operation. The working smarter approach embraces technology, such as robotic sample preparation, and faster HPLC techniques which shorten run time, and allows for more sample analysis in a given time period. The business driver will demand a more efficient operation, with fewer head count, and this driving force for a more efficient operation will continue, to an even greater degree into the post approval/commercial phase.

One of the final key deliverables in late phase development will be the extensive stability testing that will be conducted on the final market image formulation. These studies generally conducted on the process validation batches, will be utilized to justify the expiration dating of the final product. Since the HPLC assay purity method will be the key analytical tool for the analysis of these stability studies, any errors in the HPLC method could have a dramatic effect on the stability study and therefore the expiration dating of the final product. An HPLC assay at the initial point that is falsely high (102% vs. correct value of 100% label claim) will have a great deal of statistical leverage on the stability trend, that can result in shortened expiration dates. A shortened shelf life could have a major impact on the economic potential of a drug product.

V. POST APPROVAL

Following the approval of a NDA, the responsibilities for the maintenance of the analytical tools are generally transferred to the QC laboratory. In general, if the methods have been developed and validated properly, very little analytical maintenance will be necessary. The methods simply have to be run as is, and batches of product released in accordance with the relevant specifications. There are three reasons why the analytical methods may need to be changed following approval.

The first is the presence of new impurities that become evident in the finished product, that were not present in the product during development. This may be the unintentional result of a "minor" process change, or the result of a change in raw material supplier. The analytical method may need to be modified to effectively separate, identify, and quantitate the new impurity.

The second reason to change an analytical method, post approval, is because the regulatory requirements have changed over time. One example is the system suitability requirements for HPLC analysis. Over the last 20 years these requirements have changed considerably. A system suitability method from 20 years ago, would likely not comply with

today's expectations. As the regulatory requirements change, existing methods must be updated to comply with these changes.

Finally, the third reason to change an analytical method is that the scientific advances make the original technology that was utilized obsolete. At a certain point, the analytical methodology (column chemistry for example) will become obsolete. Twenty five years ago, 10 μm particle size was the preferred particle size for most HPLC methods.[24] Today, a 10 μm particle size stationary phase would not be able to provide sufficient resolution to provide the separations that is possible with more modern columns. There should be a procedure in place where the analytical methods in the QC laboratory are periodically reviewed, to determine if they still meet up to the analytical standards that are being used in the industry.

Some QC laboratories may have a separate group within the QC laboratory that will handle changes to analytical methods. Other companies will kick the method back to R&D for updating, in which case the new method will be re-transferred to the QC laboratory following validation.

There are some other areas where HPLC methods will be utilized post approval. Two areas are the analysis of product complaint samples, and in the analysis of counterfeit products. Following the commercialization of a drug product, product complaint samples will result and a complete investigation of such samples is necessary. One of the first destructive tools for such a sample (non-destructive tests such as microscopy and/or non-destructive spectroscopy should be used first) should be the assay purity test that released the product. This test will tell the scientist a number of things, it will identify the active ingredient (retention time of peak), it will show if the product has the right amount of active ingredient, and it will demonstrate if any degradation products are present at higher than expected levels in the sample. A common occurrence (especially for controlled substances) is that the product that was prescribed to a patient is removed from the container by a third party, and a different product is substituted in its place. When the unaware patient takes the dosage unit, either no effect, or an undesirable effect of the substituted product is observed. This sort of diversion will be easily clarified with the HPLC assay purity method for that product, and if coupled with mass detection, the identity of the substituted product can be realized. Additional issues that can lead to product complaints, result from improper storage (in hot/humid environments) in which case a stability indicating HPLC method could resolve degradation products consistent with such storage.

Finally, the assay purity technique can be utilized to investigate counterfeit drug samples. Counterfeit pharmaceuticals are a problem that has grown considerably in the past five years.[25] These fake products jeopardize the safety of the consumer, as well as the reputation of the

legitimate supplier. As a result, most pharmaceutical companies have gone to great lengths to assist in the elimination of this illegitimate practice. When a suspected counterfeit pharmaceutical sample is discovered, either through the report of an adverse event, or by means of the activities of law enforcement, the origin of samples will often be investigated by the pharmaceutical company. This testing may be conducted in the QC laboratory, by a special forensic group, or by the AR&D group. Regardless of who tests it, as with the complaint samples, the assay purity sample is a great starting point, following non-destructive testing. Again the assay purity test coupled to a mass spectrometer will shed light on the actual content of the material, and potentially provide investigative leads as to its origin.

A. Driving Factors for HPLC Method Development in the Post Approval Phase

Once a product is approved there will be a very strong driving force to not change the analytical methodology unless absolutely necessary. The business requirements will call for continuation of the status quo as long as product is being sold. Additionally the regulatory requirements will not want to make changes to the methods that already have the blessing of the relevant regulatory authorities. These two drivers the regulatory and business requirements at this stage will generally dictate the course of any post approval method development, since most of the scientific needs have been met.

The regulatory requirements could demand a change, if it is determined that the current analytical methodology is no longer sufficient to provide accurate data for the release of the product. This could be because of new regulatory requirements, or new technologies that have been adopted by the industry. Once a new procedure or technology becomes the industry standard, a pharmaceutical company will risk regulatory action if they do not follow suit. This threat of regulatory action, and punitive fines should also be a strong business driver to implement the new analytical procedures, since inaction will jeopardize the profitability of the company.

In the absence of strong regulatory requirements, the business drivers will generally only allow method changes, which will increase efficiency in order to reduce the cost of operating the QC laboratory. An example of such a change is laboratory automation. We have advanced from manual injectors, to auto injectors, to fully automated robotic sample preparation with online HPLC analysis. The standard manual (hand) injector today could be validated and utilized to release drug product, or study stability, but the cost and speed of such a manual procedure would not make sense compared to the low cost of today's auto injectors. Robotic sample preparation coupled to HPLC is another area where the business would drive such implementation. These sorts of changes will be driven by the desire to reduce the cost of releasing product to market.

VI. CONCLUSIONS

In conclusion, HPLC is a flexible tool that is able to generate the majority of data throughout the drug development process. HPLC methods can be modified to meet most of the challenges demanded from the scientific, business, and regulatory driving forces. HPLC methods can be utilized by highly skilled discovery chemists to fully characterize complex unknown compounds, or by a quality control technician to release a production batch under highly controlled and regulated conditions.

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11

HPLC METHOD DEVELOPMENT FOR DRUG DISCOVERY LC-MS ASSAYS IN RAPID PK APPLICATIONS

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ABSTRACT

In a drug discovery setting, the goal is to learn as much as possible about many compounds of interest in a short time. Among many criteria, obtaining experimental pharmacokinetics (PK) data from laboratory animals early in the discovery is critical to evaluate a drug candidate. Thus, a fast turnaround time from sample receipt to the PK report is crucial. Developing an accurate and fast analytical method for measuring the concentrations of a compound in plasma or tissue is the first step in order to yield the PK of a compound. The focus of this chapter is to give a practical process on how to rapidly develop a very reliable bioanalytical method when using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) in support of in vivo PK screens and studies. Some detailed discussions are provided in the area of how to choose HPLC mobile phase, HPLC column, and different sample preparation strategies. Some common issues in terms of matrix effect and background interference are also presented and the possible solutions are given. Four levels of assay criteria are suggested in order to meet the needs at the different stages in new drug discovery.

I. INTRODUCTION

Drug discovery is the process of generating compounds and evaluating all of their properties to determine the feasibility of selecting one new chemical entity (NCE) to become a safe and efficacious drug. Pharmaceutical companies are under ever-increasing pressure from both their business community and the patient population to increase the rate of the discovery of drugs. The rapid increase in the rate at which NCEs can be synthesized by medicinal chemists and tested, using various in vitro and in vivo drug metabolism and pharmacokinetic (PK) models have resulted in thousands of compounds per year undergoing the lead selection and optimization process in major pharmaceutical companies.

While medicinal chemists will continue to search for in silico programs and in vitro techniques to predict animal and human PK, the need to obtain experimental PK data from laboratory animals early in the discovery paradigm is still paramount.[1,2] PK properties are important decision-making criteria for selecting drug candidates in early drug discovery programs. A key parameter in drug metabolism and PK is the plasma concentration of the new drug after the administration of the new test compound to laboratory animals.

For scientists involved in the bioanalytical component of drug discovery, the primary ongoing challenges are speed and effectiveness of assay methods that are used in the screening of these drug candidates. A fast turnaround time from sample receipt to the PK report provides one important set of information about a potential lead drug – often producing "go/no go" feedback to chemists, while utilizing a minimum amount of resources.[2–4] On the other hand, assay effectiveness is critical for all analytical work as well.

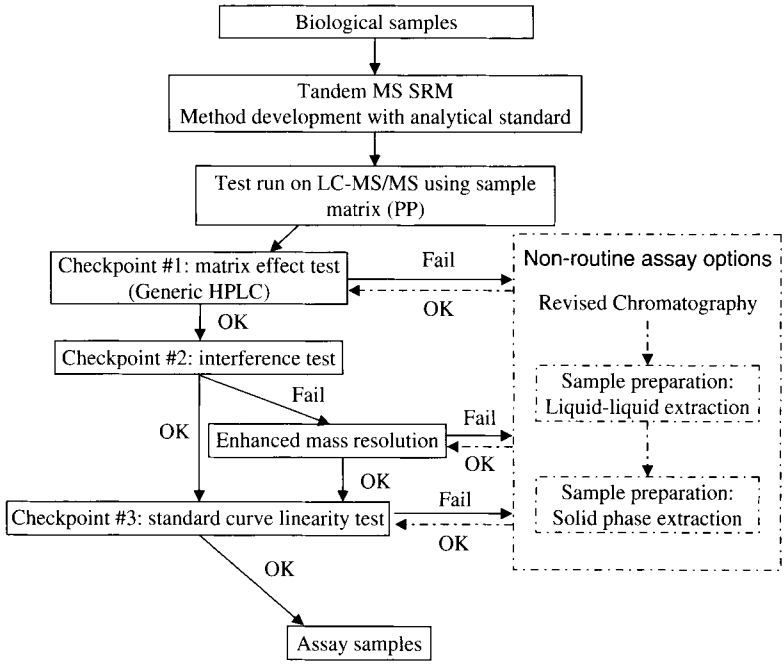

FIGURE 1 Fast method development paradigm in drug discovery. (Source: With permission from reference 7.)

Due to its inherent selectivity and sensitivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has proven to be both fast and accurate as a tool for bioanalytical assays and is now the analytical tool of choice for most pharmaceutical companies.[1–6]

It is important to consider the whole process from start to completion when trying to determine how best to decrease the time it takes to get a bioanalytical method developed. Xu et al. showed a paradigm for developing a fast and reliable assay in a drug discovery setting (Figure 1).[7] Six major steps are discussed in the report. The ultimate goal is that one can deliver a bioanalytical method with high reliability in a timely manner to fit the need of the drug discovery stage. This chapter provides more detailed discussions in these areas.

II. TANDEM MS SELECTED REACTION MONITORING (SRM) DEVELOPMENT

Because of the need to have an analytical method that can be used with multiple entities, the method should have high selectivity, ideally only responding to the compound of interest. This high degree of

selectivity would also permit the sample analysis time to be very short (1–3 min), and thus the selectivity of the method not only directly impacts the speed of analysis time but also the time required to develop the method.

The highly specific nature of the SRM experiment with the triple quadrupole mass spectrometer was first noted by Brotherton and Yost[8] in 1983. The basic analytical principle that Brotherton and Yost described in their landmark article[8] was that the multiple stages of selection in the MS/MS system reduced the noise faster than the signal, thereby creating a net improvement in the signal-to-noise (S/N) ratio. Therefore, the SRM method typically provides very high selectivity for bioanalytical quantitation.

Atmospheric pressure ionization interfaces, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), are ideally suited for interfacing with tandem mass spectrometry. Both typically produce protonated molecules, $[MH]^+$ ions, in the positive mode, with very little, if any, fragmentation. This places all of the current from the analyte into only one ion, which is an ideal situation for tandem mass spectrometry experiments. The rationale for choosing one interface over the other is most often based on the solution-phase proton affinity of the analyte – electrospray normally requires pre-protonated ions in solution and thus works best with fairly basic (or acidic) compounds, whereas APCI is also well suited for the analysis of more neutral compounds.[9]

A. Manual SRM Development

To determine the correct mass spectrometer operational conditions for a given compound, a standard solution can be introduced into the ion source by either syringe pump infusion or by multiple loop injections under a generic tune and calibration file on a triple quadrupole mass spectrometer. The choice of continuous infusion versus loop injection depends on both the type of instrument and the analyst preference. Infusion techniques require larger volumes of solution but provide additional time for parameter optimization. When performing the method development, it is recommended to "tee" in the infusion flow to the HPLC mobile-phase flow that one is going to use in an LC-MS/MS assay.

The first step in the SRM method development is to obtain the precursor ion spectrum. Next, the product ion spectrum can be found by implementing different collision energies. Typically, the most abundant product ion with its corresponding collision energy will be chosen as the SRM transition. However, the product ion chosen should represent a significant loss (e.g., greater than 20 Da) from the precursor molecule. For example, loss of water is generally not very compound specific compared to loss of a bigger

group or ring from the molecule. Typically, in the positive ionization mode, the $[MH]^+$ ion is selected using the first quadrupole (Q1), the $[MH]^+$ ion is then focused into the collision cell (q2) where it is fragmented using collision-induced dissociation (CID) into various product ions; one of the product ions is selected using the third quadrupole (Q3) and only ions of that specific *m/z* are sent to the detector.

B. Automated SRM Development

Due to parallel synthesis and high throughput screening techniques, many NCEs are tested in the drug discovery stage. These approaches have resulted in the need for the rapid development of bioanalytical methods for a large number of compounds. Since it is an extremely time-consuming process to manually infuse the compound and optimize each parameter individually, the development and optimization of quantitative LC-MS/MS-based methods for large numbers of structurally diverse compounds on triple quadrupole mass spectrometers is one of the potential bottlenecks in bioanalytical assays. Several software packages are commercially available to assist in this time-consuming task.

AutoScan™, which is provided by PE Sciex, can optimize both the ionization polarity and the MRM conditions and use a parallel injector to enhance throughput.[10] Higton et al.[11] also have shown that one could use flow-injection analysis and AppleScripts™ to create SRM methods. The procedure determines the precursor ion, optimizes the orifice potential and determines the product ion and collision energy. The SRM method is then automatically built for sample analysis.

QuanOptimize™ is the automated method development tool available on the Micromass system. Micromass has also introduced the ESCi™ multi-mode ionization source that is a combination of ESI and APCI that can alternate quickly and switch between both positive and negative polarities. There is an advantage of using these two tools together in the automatic method development process. Multiple Ion Mode ESCi™ can automatically optimize multiple compounds in either ESI (+/−) or APCI (+/−). One can also select the desired minimum fragment size for product ion inclusion. The final optimization report is generated in a text format showing the transition of each compound, ionization mode, cone voltage and collision energy.[12]

QuickQuan™ was recently introduced by ThermoFinnigan to use on TSQ Quantum triple quadrupole mass spectrometers for automated SRM method development. As the first step, the molecular weight or empirical formula, charge state and number of requested product ions for each analyte are either input manually or imported via a Microsoft Excel file. This information is stored in the compound database and can be recalled at a later time. The software then prompts the operator to

select an instrument tune file as the starting point for optimization and to choose different LC conditions or solvents for positive or negative polarity in the ESI mode. Sample introduction can occur either by loop injection (one injection port) or infusion (preferably two injection ports) by a LEAP CTC autosampler. The software then automatically performs iterative steps to optimize the MS/MS SRM method for each analyte: positive or negative polarity is selected, ion optic settings such as tube lens for the precursor ion are determined, collisions energies are evaluated and MS/MS transitions using the complete MS/MS spectra are compiled. The proposed MS/MS methods are stored in a database and a PDF file containing the relevant information is compiled. To support high-throughput assays with structurally diverse compounds, the software permits the user to group compounds together into drug sets for multiple-compound tuning and data acquisition. Furthermore, the software has the option to set a threshold for minimum intensities in a specific ion mode that can be used to identify compounds that might be better analyzed using APCI instead of ESI.[13]

Developing an SRM method using these automated procedures takes less than 3 min per compound compared to at least 15 min manually. The method sensitivity obtained from the software is usually comparable to manual optimization. The software permits MS/MS method development in an unattended fashion resulting in significant time savings.

III. HPLC METHOD DEVELOPMENT

A. Mobile Phase Requirement

Water, methanol and acetonitrile are the most common solvents used as mobile phases in LC-MS. In positive ion mode, methanol has been shown to deliver 10–50% better sensitivity than acetonitrile, while in negative ion mode there is little difference in sensitivities for most analytes.[14] Ethanol, isopropanol and tetrahydrofuran are less frequently used for the mobile phase.

Higher organic composition is desirable in LC-MS due to improved effluent evaporation at a given temperature. Ammonium acetate or ammonium formate buffers are frequently used in the mobile phase with a practical range of 0.1–20 mM. Formic acid or acetic acid (0.1–1%, v/v) are also very popular for adjusting the pH of the mobile phase.

Some additives including detergents, surfactants, ion pairing agents, inorganic acids (e.g., sulfuric, phosphoric, hydrochloric, sulfonic acids), non-volatile salts (e.g., phosphate, citrate, carbonates) and strong bases will greatly decrease the detection of analyte ions due to significant ion suppression. These compounds can also contaminate the ion source and cause it to malfunction. Therefore, they are strongly discouraged for use in the mobile phase.

TABLE 1 Column and Flow Rate Parameters

Column i.d. (mm)	Flow rate
Capillary	<10 μL/min
1.0	40–50 μL/min
2.1	0.1–0.5 mL/min
3.0	0.5 mL/min
4.6	1.0 mL/min

Source: With permission from Cohan et al., Marcel Dekker, ISBN: 0-8247-0607-2,p. 132.

B. Choosing an Appropriate HPLC Column

Column manufacturers have developed a wide range of column bore size, length, and column packing particle size to accommodate the modern LC-MS application needs. Table 1 lists the general column bore size and appropriate flow rates. Since ESI and APCI can be operated at flow rates as high as 1 and 2 mL/min, respectively, most of the columns are compatible. For hydrophobic compounds, the traditional C_{18} column allows selectivity for polar versus non-polar compounds. Shorter chain packings such as C8 or C4 present fewer hydrophobic surfaces for interaction with analyte molecules. Aromatic compounds may be well suited for analysis by phenyl columns. Cyanopropyl columns allow the greatest degree of separation for polar compounds.

Due to the high sensitivity and selectivity of LC-MS, less resolution and selectivity are required from the LC separation. Typically, high-organic mobile phases are used to quickly elute the analytes. To gain adequate retention with this kind of mobile phase, highly retentive columns should always be selected initially.

C. Ultra Performance Liquid Chromatography (UPLC)

As particles decreased from the 10-μm size in the 1970s to the 3-μm in the 1990s, the throughput and resolving power of HPLC columns has increased significantly. Recent technological advances have made 1.7-μm particle size packing material available.[15] Coupling with high-pressure pumps (>10,000 psi, ACQUITY™ HPLC system, Waters Corp.) and high-speed acquisition MS (Premier™, Waters Corp.), UPLC is likely to be very useful for assaying drug discovery PK studies.

The 1.7-μm bridged-ethyl hybrid (BEH) particle size is highly uniform and has a very narrow size distribution, which improves efficiency and peak shape. Improvements in chromatographic resolution with UPLC are apparent from generally narrower peak widths (1–2 s for a 10 min

separation).[15,16] The flow rate on UPLC column is typically between 0.2 and 1 mL/min. Injection volume is 1–10 μL.

Churchwell et al.[17] showed a comparison study between traditional HPLC and UPLC. The test compounds were small molecules. They reported that sensitivity increased with UPLC. This was found to be analyte-dependent, but could be as large as 10-fold. Also, improvements in method speed were as large as fivefold under conditions of comparable peak separations. In general, UPLC provided significant improvements in method sensitivity, speed and resolution.

Swartz et al.[18] also reported that using the UPLC system, resolution and efficiency improved two to three times, and quantitative accuracy also improved significantly, at sub-nanograms per milliliter sensitivities, when compared to HPLC under the same conditions using the current 15 min inject-to-inject cycle time method. The improved UPLC peak capacity also meant impurities and other related compounds were resolved with UPLC, even for compounds that had proved difficult to separate by HPLC. A new method developed and optimized for the UPLC system at an inject-to-inject cycle time of 1.5 min maintained the original resolution and efficiencies, and a factor of 10 improvements in sample throughput, while maintaining LC-MS/MS detection capability. Solid-phase sample cleanup resulted in lower background without sacrificing quantitative recovery.

Overall, UPLC offers significant theoretical advantages in resolution, speed and sensitivity for analytical determinations, particularly when coupled with mass spectrometers capable of high-speed acquisitions. These sub 2-μm particle columns operate at pressure significantly greater than conventional HPLC columns and thus require a specially designed chromatography system to obtain maximum chromatographic performance. The sharp peaks produced by UPLC are of particular advantage when coupled to electrospray mass spectrometry resulting in superior sensitivity and hence lower limits of detection.

IV. SAMPLE PREPARATION

Sample preparation is still a topic of high importance when developing a method that uses an LC-MS/MS system to assay biological samples. It is well documented that because of the large amounts of protein present in plasma samples, conventional HPLC columns will not tolerate the direct introduction of plasma; therefore, most bioanalytical assays have a sample preparation step to remove the proteins from the sample.[1,6,19] In addition, there are other important reasons to include a sample preparation step when developing LC-MS/MS methods. These include the reduction of matrix components from the samples and elimination of ion suppression (also called "matrix effects") in the mass spectrometry response.[20] Once an

analytical method has been developed, it is desirable that method performance remains reasonably consistent over time. The results generated based on the method should be relatively free from systematic error and any relative error should be characterized and consistent and meet method acceptability guidelines.[1] Therefore, sample preparation is used to ensure that a method maintains certain basic elements of ruggedness and consistency that are expected in any bioanalytical assay.

Due to the nature of drug discovery, sample preparation procedures must be developed in a short time, with a reasonably high certainty of success. An appropriate extraction technique to remove most of the protein (>90%) from a sample is highly desirable. For drug discovery bioanalytical applications, protein precipitation (PP) is the most common sample preparation procedure, while liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have also been reported as sample preparation techniques for plasma samples.[1,3,21,22] In the following text, several important techniques that have been widely used for the drug discovery PK studies are discussed.

A. Protein Precipitation

Since simple organic solvents such as methanol or acetonitrile are effective at removing more than 98% of proteins from plasma when used in ratios of 2:1 or greater[23] and are compatible with LC-MS/MS systems, they are the most popular denaturing solvents to perform the protein precipitation for drug discovery PK studies. After addition of the denaturing solution to the plasma sample, protein precipitation is facilitated by vortexing and centrifugation of the plate.[5] The denaturation process causes the analytes to be released from the proteins and remain in the supernatant liquid. A small aliquot of the supernatant is then injected directly into the LC-MS/MS for assay. A recent research report by Xu et al.[24] suggested that using a higher organic solvent to plasma ratio (6:1) for protein precipitation would significantly reduce the level of background interference (Figure 2) and increase the signal-to-noise ratio, which eventually increases the assay sensitivity (in this case a fivefold lower limit of quantitation (LOQ) was achieved when the plasma sample went through a protein precipitation using acetonitrile to plasma ratio of 6:1 instead of the more common 3:1 ratio).

Although the protein precipitation procedure is simple and has become a very useful sample preparation tool, there are some possible issues of concern. Protein precipitation is ineffective at removing lipids from samples, which can buildup on the column head and eventually clog and change the retention characteristics of the column. It is also ineffective at removing salts from the samples, which can cause matrix effects in the MS response. Therefore, it is recommended to use a guard column (to capture lipids) and post-column divert valve (to avoid salts)

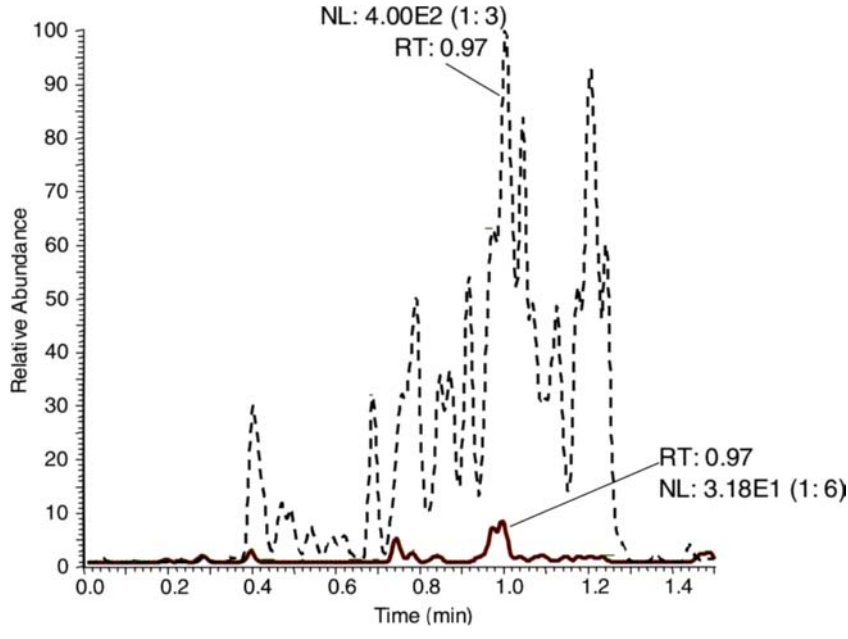

FIGURE 2 **Normalized mass chromatograms of blank control plasma obtained using protein precipitation ratios of 1:3 and 1:6. (Source: With permission from reference 24.)**

in LC-MS/MS systems when the biological samples are processed by protein precipitation.[19]

Due to the large numbers of compounds and the fast turnaround required in the early drug discovery stage, automated sample preparation is highly desirable. A semi-automated protein precipitation procedure was reported by O'Connor et al.[25] using a Beckman Biomek 2000 liquid-handling workstation. The method included preparation of dilutions of analyte stock solutions, spiking these into control plasma to generate analytical standards, and preparation of sample suitable for analysis by LC-MS/MS by precipitation of plasma protein with acetonitrile, centrifugation and dilution of the supernatants with HPLC buffer. All of these steps, apart from centrifugation, were performed without manual intervention on the workstations using 96-well plates. Other groups[5,26] have reported using the Tomtec Quadra 96 Model 320 liquid handling system to process protein precipitation in a semi-automated format. Using these systems, one analyst can extract up to 96 samples in approximately 20 min. These semi-automated protein precipitation procedures have greatly facilitated the analysis of small sample sets for a large number of analytes, a situation regularly encountered in an early drug discovery PK studies.

B. Liquid–Liquid Extraction

LLE is based on the principles of differential solubility and partitioning equilibrium of drug molecules between aqueous (sample) and organic phases. Basically, an immiscible organic solvent is added into the plasma sample, followed by vortexing to facilitate equilibrium partitioning of analyte molecules between phases. When the phases are separated, the organic phase is evaporated to dryness and reconstituted with mobile phase or some other solvent system and then injected onto the LC-MS/MS system. One advantage of LLE is the freedom of choosing an extraction solvent with a polarity that matches the analytes of interest. The best recovery is obtained when the organic solvent versus sample volume is greater than threefold.

This technique is simple to implement, however, it is quite labor intensive. Automated sample preparation is highly desirable, not only because of time savings, but in order to relieve the tedium associated with processing large numbers of samples. Some semi-automated LLE procedures were introduced by utilizing commercially available workstations for liquid handling in a 96-well multi-channel plate format.[27–29] However, full automation was not achieved until recently.[30,31]

C. Solid-Phase Extraction

SPE generally provides the cleanest extract of all sample-preparation techniques in terms of selectivity. The price paid for this performance is that method development is generally the most complex and time consuming. The most popular SPE devices are pre-packed cartridges and membrane disks.[32,33] There are a few steps involved in using the cartridge or membrane disk. The initial step is typically conditioning of the sorbent bed with a small volume of methanol or acetonitrile and water or buffer. Then the sample is loaded onto the sorbent and the sample solvent is pulled through by vacuum or centrifugal force. A series of wash steps are then implemented using different solvents with increasing polarity to remove salts and some matrix components. The final step is to elute the compound with a strong solvent. The elution solution can either be directly injected into the LC-MS/MS or dried down first and reconstituted and injected later.

Due to the complexity of this process, automation of the SPE procedure is critical in the drug discovery stage. The most popular automated SPE procedures are setup in a 96-well format using 96-well workstations.[34,35] Compared to individual sample processing in series, using the 96-well format in a parallel sample processing procedure can dramatically improve the efficiency and reduce the sample assay turnaround time. Another application of automation is the on-line SPE approach.[36] The disadvantage is that the throughput using on-line SPE approach is

usually lower than the 96-well format procedure and requires a fairly complex instrument setup (e.g., multiple columns and switching valves).

In addition, no matter which approach is going to be used for method development, there are several issues that need to be tested before an SPE procedure can be used routinely. These issues include recovery, assay precision and carryover potential.

D. Direct Injection

Due to the fast turnaround request in the drug discovery stage, traditional sample preparation is often the slow step in the method development procedure. An alternative approach is direct injection analysis. Since there are large amounts of protein present in plasma samples, conventional HPLC columns will not tolerate the direct introduction of plasma. Several new packing materials have been designed to use in direct sample injection devices.

High flow rate liquid chromatography or turbulent flow chromatography-tandem mass spectrometry typically involves a dual or even ternary-column configuration.[37–40] The extraction columns are normally made of a mixed hydrophobic–hydrophilic polymer phase with a larger particle size. The plasma samples will be loaded onto this extraction column with a high flow rate (e.g., 4–10 mL/min) with 100% aqueous mobile phase followed by elution onto a conventional analytical column at a regular flow rate (e.g., 1 mL/min).

Restricted access media (RAM) are designed as packing material for direct extraction of small molecules in biological samples. The separation between small molecule and macromolecule is based on both particle size and chromatographic interaction. Large macromolecules such as protein, which are unable to penetrate the hydrophobic pores, are first eluted to waste. Small molecules such as drugs penetrate the pores and are retained and separated by conventional retention mechanisms. Another type of column called the monolithic silica column has also been reported to be used for direct plasma analysis.[41] The monolithic silica column has a novel stationary phase with small-sized skeletons and large through-pores to simultaneously reduce the diffusion path length and flow resistance relative to a traditional, particle-packed column.[42,43] It can be operated at higher flow rates (e.g., 4–8 mL/min) without a concern for the back-pressure. Even though a single column method can be used,[44] these columns usually require an additional analytical column for chromatographic separation in combination with a column-switching technique in a coupled-column mode due to matrix effects.[45,46]

In comparison, PP is the simplest approach to implement because it requires no method development, and removes the majority of the protein from the sample. SPE is a very effective method for removing matrix components and often provides the cleanest extract. The price paid for

this performance is that SPE method development is generally the most complex and time consuming and typically involves multiple method optimization steps. LLE has also been used extensively to remove unwanted matrix components. It offers reasonably easy method development and is an effective sample cleanup process. Although LLE is generally easier to implement than SPE, it is still more labor intensive than PP. Direct sample injection techniques typically need complex analytical configurations, which limits their usage. Therefore, protein precipitation is still the most popular sample preparation method to handle biological matrices (e.g., plasma) in the drug discovery stage.[1–3,5] The ability to use PP in the sample preparation step is largely due to the high selectivity of the LC-MS/MS system when used in the SRM mode, i.e., there is typically little or no response from any compound other than the analyte of interest. In essence, the mass spectrometer is being used for the bulk of the "separation" process, and the LC is serving in no small part as an automated sample introduction technique. Thus, sample cleanup time is minimized by use of the PP technique. Method development time is also reduced from weeks to less than 1 day.

V. MATRIX EFFECTS

One of the challenges for bioanalytical method development using LC-MS/MS is the need to understand the possible "matrix effect" problems (ion suppression or ion enhancement), which can result in significant errors in quantitation if careful attention is not given to avoid this problem;[1,20,47–49] otherwise, undetected matrix effect problems could lead to significant errors in the calculation of PK parameters.

In FDA guidelines for bioanalytical validation, matrix effects are defined as "interference from matrix components that are unrelated to the analyte."[50] This broad definition includes both ion enhancement and ion suppression. These effects can be caused by ionization competition of co-eluting components within the atmospheric pressure ion source.[20,47] Compared to ion enhancement, ion suppression is more problematic in that it will reduce the sensitivity of the assay, and it is a more common problem. If one cannot control the variability caused by matrix effects, both ion enhancement and suppression can be challenging, since both will result in poor reproducibility of results.

A. Source of Matrix Effects

Since fast turnaround time is required regardless of whether the assay is easy or difficult, identifying the source of matrix effects becomes crucial. It is generally believed that ESI is more subject to matrix effects than APCI. However, it has been found that different instrument interface designs

could also affect the tolerance of matrix effects on the different ionization modes. A comparative study using three different triple quadrupole mass spectrometers (PE Sciex API 3000, Micromass Quattro Ultima and Thermo-Finnigan TSQ 7000 API-2) to evaluate their sensitivity toward matrix effects was reported by Mei et al.[51] Significant ion suppression was observed when using the Micromass Quattro Ultima system with both ESI and APCI ionization modes and using the Finnigan TSQ system with ESI mode, while no ion suppression was observed using Sciex API 3000 with either APCI or ESI mode. Xu et al.[52] also reported some matrix effects observations on the MS instruments from different vendors when different dose formulations were investigated. For early eluting compounds, significant ion suppression was observed on the Finnigan Quantum in both the ESI and APCI modes, the Micromass Ultima in the ESI mode and the Sciex API 3000 in APCI mode when 0.1% Tween 80 was used in the dosing formulation. When PEG400 was used in the dosing formulation, all three vendor's MS showed significant ion suppression in the ESI mode for the early eluting compounds. APCI in this case showed no significant matrix effects. These compelling data suggest that matrix effects in HPLC-MS/MS assays are not only ionization mode (APCI, ESI) dependent, but can also vary between different vendors' source designs.

In addition to instrumental parameters, the most important factor that determines ESI responses is the physical and chemical nature of the analyte and co-eluting components. Identifying the nature of matrices will provide useful information for overcoming the matrix effects. Most drug candidates are small molecules with log *P* ranging from 1 to 5 and are retained on a reversed-phase HPLC column.[53] Hydrophilic or polar constituents are not an assay problem for relatively hydrophobic NCEs since they can be easily separated on an HPLC column. Some hydrophobic components that are eluted in the later retention time of a reversed-phase chromatogram have a high potential to co-elute with pharmaceutical compounds and can lead to matrix effects.[54] While these types of matrix issues are relatively challenging, they are still manageable by carefully separating these components from analytes of interest using either various sample preparation techniques or HPLC adjustments. The most difficult matrix effect problems are those caused by hydrophobic components existing in relatively large amounts and with retention times that overlap the analytes.[55–57] In this situation, the chromatographic system does not provide sufficient separation, resulting in significant ion suppression for compounds that elute in that part of the chromatogram.

Many studies have reported that matrix effects are mainly due to endogenous components (e.g., salt, amines, fatty acids, triglycerides, etc.) in biological fluids.[58,59] However, Mei et al. reported that matrix effects can also be caused by exogenous materials, such as polymers contained in different brands of plastic tubes, or Li-heparin, a commonly used anticoagulant.[51]

Other exogenous materials that have potential matrix effects are dosing excipients. Multiple studies have demonstrated that excipients such as PEG400, propylene glycol and Tween 80, used for either intravenous or oral formulation, can cause significant matrix effects in both the ESI and the APCI modes.[52,60,61] Another unique aspect of this type of matrix effect is that it is "variable" with time, since these dosing excipients will also undergo an absorption, distribution and elimination process in animals, which will be reflected in the plasma samples collected at different time points.[60–62] Formulation matrix effects that are time-course dependent are generally harder to detect and eliminate. Even though techniques such as appropriate sample purification or employing negative ionization mode can diminish the problem, avoiding such excipients is a safer alternative.[52,60] If one cannot avoid these dosing excipients, then it is important to evaluate their effect, if any, on the LC-MS/MS system that is used for assaying the samples.

Many studies have also proven that for ESI, the intensity of ion signal is dependent on not only the chemical nature of the analyte but also many other factors, including the presence and concentration of electrolytes in the liquid,[23] volatility of the solvent,[30,31] surface activity of the droplet,[25,27–29] presence of non-volatile components,[5,26] flow rate of electrosprayed solution,[24] concentrations of other ionizable species[27,37] and competition of gas-phase ion-transfer reaction between analytes and other ionized ions.[30,31,38,39] The possible mechanisms for ion suppression are competition for limited surface excess charge, incomplete evaporation, ion pairing or competition for protons in the gas phase.[47]

B. Evaluation of Matrix Effects

Due to the importance of the matrix effect potential in an LC-MS/MS assay, its evaluation is the first step. Several approaches have been developed to evaluate the matrix effects using different experimental techniques and each has its own advantages and disadvantages.[47] Post-column infusion[20,47,48,63] and direct comparison of mass response in different matrices using pre-spiking or post-spiking approaches[58,64] are the most common procedures. The purpose of the evaluation is to locate the portion of the chromatogram that shows a matrix effect and to know the relative hydrophobicity, to identify the source of matrix effects (if possible), to select appropriate internal standards and to compare the effectiveness of various separation procedures.

In order to directly observe the location of ionization suppression in an LC-MS/MS assay, Bonfiglio and colleagues[65] developed a post-column infusion scheme that has been widely adopted by many laboratories. Basically, control sample extracts are injected on the HPLC column with generic gradient conditions, while a constant amount of analyte is infused into the HPLC eluant before it enters the mass spectrometer. Any ion suppression is observed as the variation of MS response of the infused

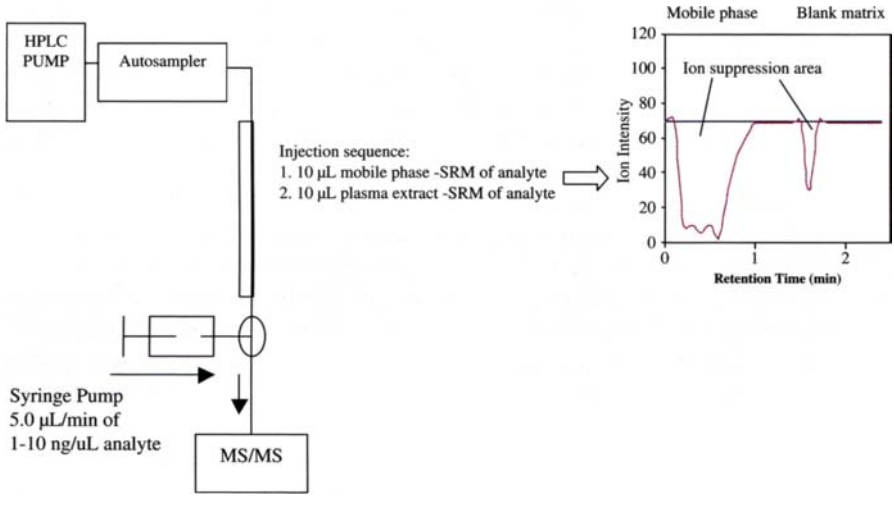

FIGURE 3 **Evaluation of matrix effects using the post-column infusion technique. (Source: With permission from reference 47.)**

analyte, as compared to the response from the injection of blank mobile phase (Figure 3). Even though this method has the advantage of showing the region in the chromatogram of ion suppression, it only provides a semi-quantitative picture of matrix effects that are not easy to tabulate or graph for comparison when many different matrices with a large number of compounds are studied. In addition, since the post-column infusion is usually performed at relatively high concentrations, it can contaminate the source, generating a high background signal and reducing sensitivity; when this happens, instrument cleaning must be conducted to solve the problem.[66]

When there are multiple compounds or multiple matrices that need to be evaluated, the most efficient and straightforward method to detect matrix effects is the direct comparison study. A set of samples containing the same amount of analytes and internal standards in matrix-free solvent, blank matrix used to prepare calibration standards and blank matrices obtained from different sources or pre-dose blank sample plasma are tested under the same assay condition. If the MS response in pre-dose blank sample plasma is within 25% of the MS response in standard plasma, then this method is considered free of matrix effects and can be used for quantitation in drug discovery studies. For example, this method was efficiently utilized to compare the matrix effects among three different tandem mass spectrometers and to investigate the endogenous and exogenous sources of matrix effects.[51,52]

In order to separate the recovery loss from matrix suppression, one can use the pre-spiking and post-spiking approach, where pre-spiking

refers to adding standards and internal standards before sample preparation and post-spiking refers to adding standards and internal standards after sample preparation.[64] This technique is especially helpful when complicated sample preparation procedures, such as SPE and LLE, are used, since these procedures, unlike protein precipitation, are more subject to analyte loss (low recovery).

Another important matrix effect evaluation procedure for discovery studies is to use samples obtained from the tested animals. In the past, the pre-dose plasma sample was considered to be the most appropriate sample for matrix effect evaluation for PK studies. However, based on the recent research report by Xu et al.,[52] this approach cannot guarantee that matrix effects will not be an issue. Since the matrix effects from dosing recipients, e.g., Tween 80 and PEG400, gradually diminished with time post-dose, using a single plasma sample collected at one time point from Tween 80 or PEG400 vehicle-dosed animals to generate a calibration curve will not correct for ionization suppression of the real plasma samples from the PK study. Even using plasma from a control (vehicle-dosed) animal and pooling multiple post-dose time points for the calibration curve will not correct this matrix effect problem because the matrix ion suppression varies with the post-dose collection time. Therefore, pre-dose samples cannot provide sufficient information if the dosing excipients are the cause of the matrix effects. In these situations, one can consider using the so-called "standard addition" method to evaluate matrix effects. Basically, the unknown sample (X) and X with an added known amount of analyte (X + A) are analyzed along with calibration standards under the same processing procedures.[47] Using the calibration standards, one can obtain the observed concentration of X and X + A. The matrix effects can then be evaluated by comparing the observed X + A with the expected X + A. If the difference is greater than 25%, then it can be considered to be due to matrix effects. This method was successfully used to quantify toxins in scallops where the degree of signal suppression varied from scallop to scallop.[67]

Different strategies need to be used for solving matrix effects with different causes and sources. For a small amount of endogenous hydrophobic matrices, one can modify the chromatographic separation conditions or try other sample preparation procedures, such as off-line or on-line SPE or LLE. For matrix effects caused by the exogenous materials, e.g., dose excipients like Tween 80 or PEG400, it is recommended to replace them with hydroxyproyl-β-cyclodextran or methyl cellulose whenever it is possible.[52] Li-heparin should be avoided as the anticoagulant for plasma samples to be assayed by LC-MS/MS, especially in those laboratories that support drug discovery studies. The alternative anticoagulant is Na-heparin or Na_2-EDTA, which has not been reported to show significant matrix effects.[51] Based on the concept that the extent of matrix effect is dependent on the amount of matrix in the ion source,

introducing the minimum amount of sample by injecting small sample volume, using a divert valve or diluting samples with blank standard plasma can be the practical approaches in drug discovery. When all else fails, try a different ionization mode or a different brand of instrument in order to avoid matrix effects.

VI. BACKGROUND INTERFERENCE: ENHANCED MASS RESOLUTION STRATEGY

Another challenge in LC-MS/MS method development is that the limited sample preparation and fast HPLC leads a higher chance for target-interference co-elution;[68–72] this is becoming more common as one strives to get to lower LOQs. In some cases, these interferences from the endogenous matrix are not eliminated when using SRM and unit mass resolution on the mass spectrometer. One approach to overcoming this problem is to achieve maximum chromatographic resolving power within a short chromatographic time. However, HPLC method development can require a lot of effort and can be quite time consuming. Another possible approach to improve the measurement of the analyte is to increase the mass resolving power of the MS.

Mass resolution, like sensitivity, is commonly used as a performance specification for an MS instrument and varies greatly depending on the mass spectrometer analyzer and the detailed design components of a particular instrument. The mass resolution of a mass spectrometer is qualitatively defined as its ability to discriminate between adjacent ions in a spectrum. Mass resolution is often defined as a function of mass and is given by the following equation: $M/\Delta M$, where M is the mass of the ion and ΔM is the smallest increment of mass that can be distinguished by the analyzer. The unit for mass is daltons (Da) and ΔM is often determined using the full width at half maximum (FWHM) definition of the mass peaks.[73]

Achieving good mass resolution and peak shape is complex. As a general rule, the more restrictive the conditions for allowing ions through the analyzer, the fewer the number of ions that will be allowed through. In the triple quadrupole mass spectrometer, the amount of sensitivity lost will depend on the resolution of the instrument. The greater the mass resolution (and narrower the peak), the greater the loss in sensitivity. However, improved mass resolution/transmission characteristics for quadrupole mass spectrometers have recently been achieved with the introduction of the TSQ Quantum® (Thermo-Finnigan) MS system.

For a triple quadrupole MS system, unit mass resolution is normally defined as 0.7 Da using the FWHM definition.[74] Figure 4 shows a test compound's partial mass spectrum (Q1 scan) under different mass resolution settings (Q1 at 0.7, 0.45, 0.2, 0.1 and 0.07 Da FWHM) on a TSQ Quantum instrument. The relative abundance of the ion decreased when the mass resolution increased. At a peak width of 0.2 Da FWHM, the

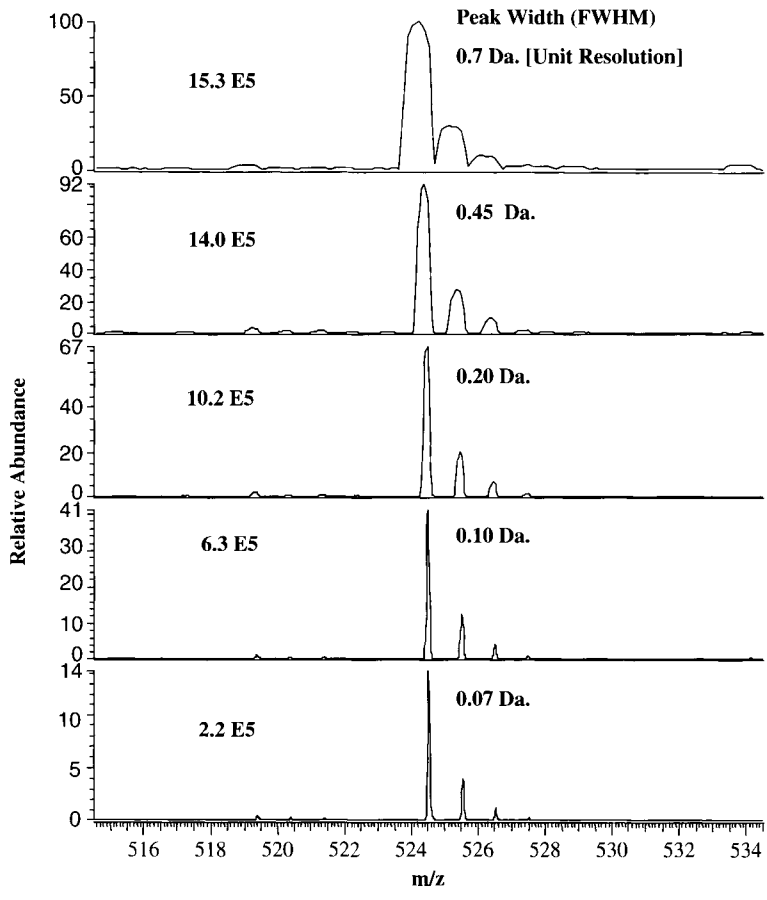

FIGURE 4 **Mass spectra of a candidate compound (Q1 full scan). (Source: With permission from Thermo-Finnigan.)**

relative abundance of the ion (10.2E5) was only reduced by about 33% relative to the abundance (15.3E5) at the unit mass resolution setting, 0.7 Da FWHM. At a peak width of 0.1 Da FWHM, the relative abundance of the ion (6.3E5) is about 40% of the one at 0.7 Da FWHM (15.3E5). When the mass resolution was also set to 0.07 Da FWHM, the relative abundance of the ion (2.2E5) decreased significantly (now only about 14% of the original 0.7 Da FWHM ion intensity). From this example, one can see that significant increases in mass resolution were obtained (0.2 Da FWHM) before the signal intensity dropped below 50% of the unit mass resolution setting.

Figure 5 shows a representative mass chromatogram of a drug candidate in rat plasma.[7] In this case, significant background interference to the signal of this compound was observed at the unit mass resolution setting

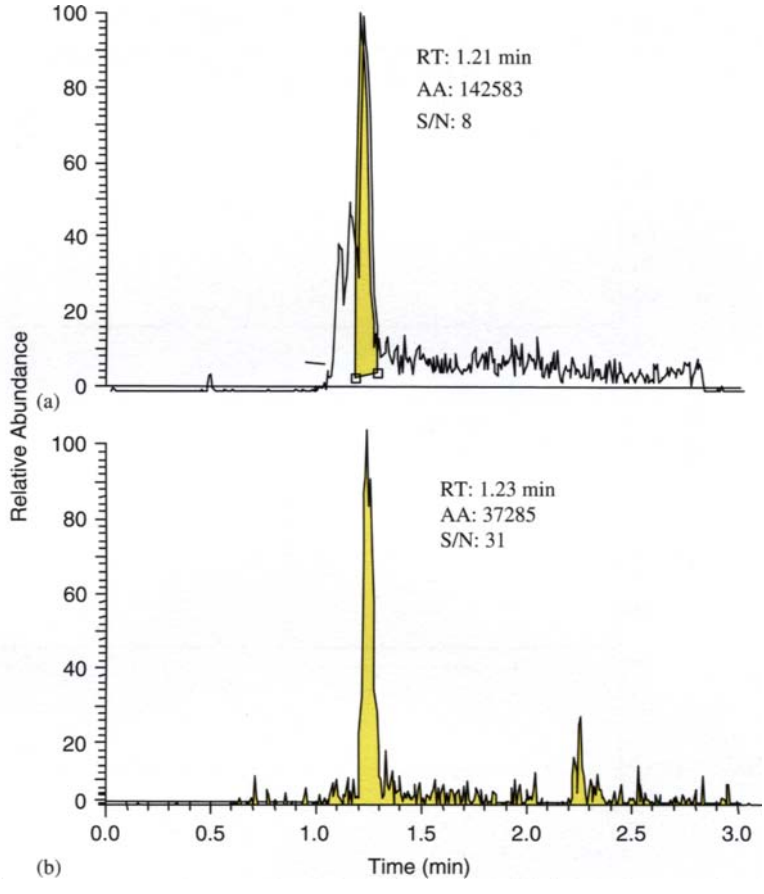

FIGURE 5 **Representative mass chromatograms of a test compound at 250 ng/mL in rat plasma at (a) 0.7 FWHM and (b) 0.2 FWHM. (Source: With permission from reference 7.)**

(Q1, 0.7 FWHM) (Figure 5a). A calculated signal-to-noise (S/N) ratio of 8 was determined. Hence, the LOQ for this compound at the unit mass resolution under this assay conditions was approximately 250 ng/mL. When the same sample was assayed at an enhanced mass resolution (Q1, 0.2 FWHM), a much cleaner SRM peak was observed (Figure 5b). This was a consequence of the removal of a considerable amount of isobaric chemical interference from the analyte through the improved mass selectivity using the enhanced mass resolution setting. In addition to a more uniform SRM peak, an improved S/N ratio of 31 was obtained. The dramatic decrease in background noise at the enhanced mass resolution setting was responsible for this significant improvement in S/N ratio, despite the loss of a factor of 3–4 in peak area for the analyte. Since the S/N ratio of 31 at

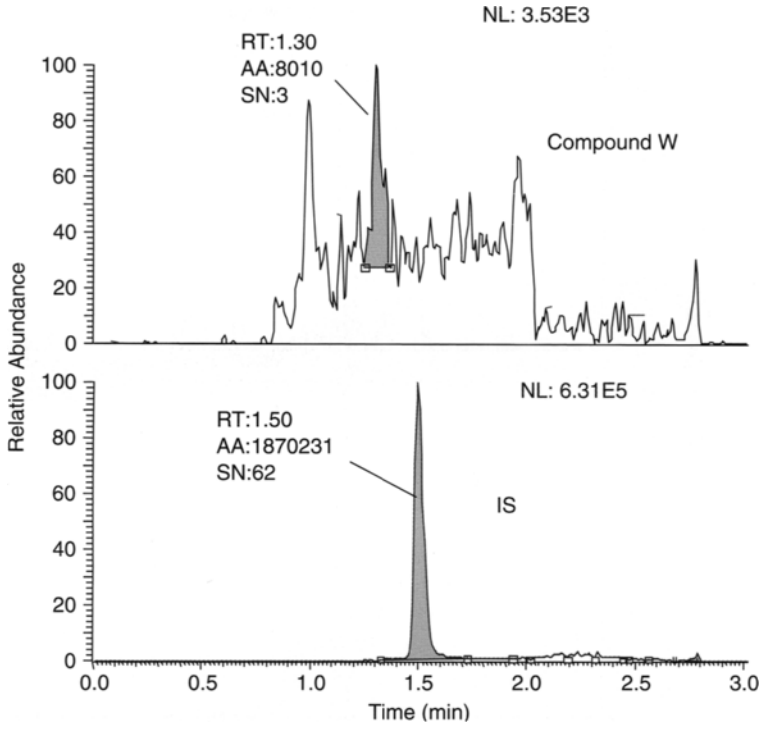

FIGURE 6 **Mass chromatograms of compound W (0.1 ng/mL) and internal standard spiked into the plasma matrix, obtained using the Quantum LC/MS/MS system with positive ESI mode under unit mass resolution (Q1, 0.7 Da FWHM). (Source: With permission from reference 75.)**

250 ng/mL was above that required for the LOQ, the assay LOQ was able to be set to 25 ng/mL under the enhanced mass resolution condition.

Background interference can be a more common problem as one tries to achieve very low LOQs.[74–76] Xu et al.[75] reported a sensitivity enhancement of twofold in both plasma and brain samples by just replacing the unit mass resolution setting to enhanced mass resolution setting. Using a Thermo-Finnigan Quantum, set to unit mass resolution (Q1, 0.7 Da FWHM) resulted in the mass chromatograms shown in Figure 6 for a mouse plasma standard spiked with compound W at 1.0 ng/mL. As shown in Figure 6, at the retention time of 1.3 min, only a small peak with an S/N ratio of 3 could been detected as the signal for the analyte (compound W). Clearly, the analyte peak was co-eluting with a lot of background interference. When the same sample was injected onto a Quantum MS system with enhanced mass resolution settings (Q1, 0.2 Da FWHM), a baseline-separated peak was observed with an S/N ratio of 7 (see Figure 7). By comparing Figures 6 and 7, it is evident

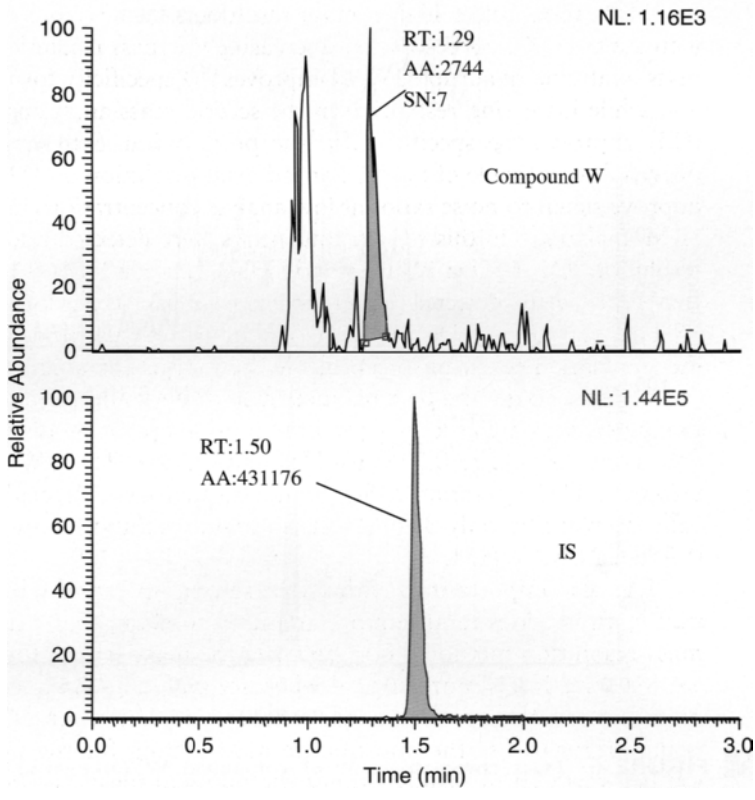

FIGURE 7 **Mass chromatograms of compound W (0.1 ng/mL) and internal standard spiked into the plasma matrix, obtained using the Quantum LC/MS/MS system with positive ESI mode under enhanced mass resolution (Q1, 0.2 Da FWHM).**
(Source: With permission from reference 75.)

that the Quantum MS with enhanced mass resolution was able to reduce the background interference so that a lower LOQ could be obtained for this compound in this matrix.

Jemal and Ouyeng[77] described the use of the Quantum MS system in the ESI mode for the determination of nefazodone in human plasma or urine samples. Both unit and enhanced mass resolution were investigated under the SRM transition that was selected (m/z 470.232 → 274.156). For unit mass resolution, Q1 and Q3 were set at 0.7 Da FWHM, and for enhanced mass resolution, Q1 and Q3 were set at 0.2 and 0.7 Da FWHM, respectively. After using protein precipitation, plasma or urine samples were injected into the LC-MS/MS system for analysis. In this report, the use of enhanced mass resolution allowed the assay to be successfully applied to a human plasma containing nefazodone at the 30 pg/mL level with a higher signal specificity (and higher S/N) than was obtained when using unit mass resolution for the same sample.

Most users select higher mass resolution for Q1, but one can also increase the Q3 mass resolution. Increasing the mass resolution of the first mass-analyzing quadrupole (Q1) improves the specificity for the precursor ion, while increasing resolution in the second mass-analyzing quadrupole (Q3) improves the specificity for the product ion. Schweingruber et al. provided an example of how enhanced mass resolution on Q1 and Q3 can improve signal-to-noise ratios at low analyte concentrations in quantitative SRM analyses.[78] In this report, four peaks were detected under unit mass resolution (Q1, 0.7 Da FWHM and Q3, 0.7 Da FWHM). However, only two peaks were detected under enhanced mass resolution (Q1, 0.1 Da FWHM and Q3, 0.5 Da FWHM). The improved specificity is evident by the elimination of extraneous peaks in the mass chromatogram.

In order to get the best balance of sensitivity and selectivity, the following settings are recommended for routine operation of the Quantum MS system: set Q1 to 0.2 Da FWHM and Q3 to 0.7 Da FWHM.[74] When using the TSQ Quantum MS system, chromatographic peak areas typically decrease by only 30–60% when mass resolution is increased from 0.7 to 0.2 Da FWHM.[74,75]

It is also important to realize that setting up enhanced mass resolution methods does require more attention to detail than setting up unit mass resolution methods. For instance, the mass setting for the precursor ion selection is more critical when setting up an enhanced mass resolution assay because the analyte mass peak is narrower.[74] Therefore, it is important to use the appropriate mass setting for the Q1 precursor ion (set to the nearest 0.1 Da, not the nominal mass) in order to avoid missing the top of the mass peak, which would lead to selecting ions from either the ascending or descending side of the normal distribution of the mass peak for the precursor ion. Furthermore, as discussed by Jemal and Ouyang,[76] the precursor ion (Q1) mass values appear to change slightly as the mass resolution (FWHM setting) is changed. Typically, a change of 0.1 Da was observed when the Q1 mass setting changed from 0.7 to 0.2 Da FWHM.[76] Therefore, for a routine use of an enhanced mass resolution, SRM-based quantitative bioanalytical method, a precursor ion scanning result obtained immediately before the start of the analysis is recommended in order to update the exact precursor ion mass in the SRM table of the bioanalytical method. It is normally not as important to recheck the product ion (Q3) mass setting since the mass resolution (FWHM) for the product ion in the SRM method is typically set at 0.7 Da.

Other research reports[78–81] have also shown the power of using the additional resolving power of the quadrupole mass analyzer (enhanced mass resolution) to eliminate background interference problems without going through a more complex sample preparation procedure or revising the chromatography conditions. As a result, this option can significantly reduce the method development time. If the interference problem cannot be solved by using enhanced mass resolution, then other assay options

(e.g., different chromatography, liquid–liquid or solid-phase extraction) can be used to eliminate the interference problem.[7]

VII. LIMIT OF QUANTITATION, DYNAMIC RANGE AND LINEARITY

One way to view the drug discovery process is that it is a series of stages through which compounds must pass in order to qualify for being a development candidate. There are multiple stages that involve measuring PK parameters. However, there have been no clear bioanalytical assay guidelines to follow in the discovery stage. Korfmacher[1] has presented a four-level strategy (Figure 8), which covers assays from the very early screening stage to the late development stage. As shown in Figure 8,

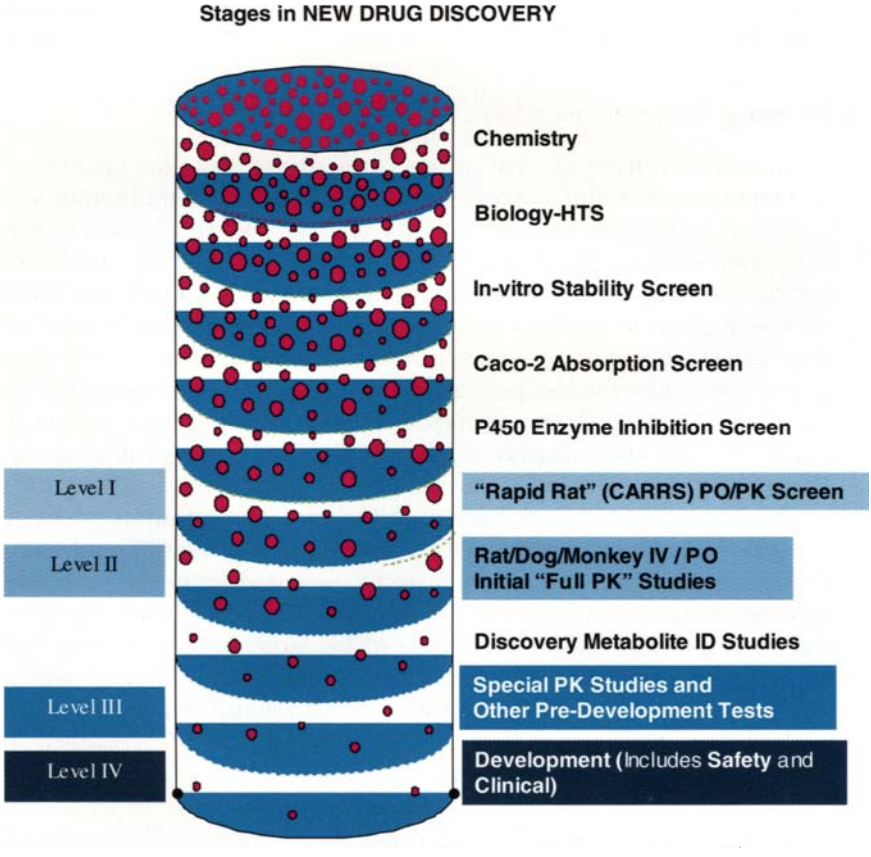

FIGURE 8 Stages in new drug discovery. A large number of compounds are screened out by each stage. The levels I–IV refer to the assay rules outlined in this chapter. (Source: With permission from reference 1 (ISBN: 0-8493-1963-3).)

TABLE 2 Assay Levels for Bioanalytical Methods

Drug stage	Assay type	Summary of major rules	GLP?
Screening	Level I	Use a two-point standard curve	No
Lead optimization	Level II	Use a multi-point standard curve but no QCs	No
Lead qualification	Level III	Use a multi-point standard curve plus QCs	No
Development	Level IV	GLP rules	Yes

GLP, Good Laboratory Practice.
Source: With permission from reference 1 (ISBN: 0-8493-1963-3).

level I is the screening stage, level II is lead optimization, level III is lead qualification and level IV is development. As the candidate compound approaches the development stage (IV), the bioanalytical assay requirements become stricter (Table 2).

A. Level I: Screening Assays Requirement

Screening can be defined as the stage where a larger number of compounds are tested in order to select a smaller number for optimization. Cassette ("N-in-1" or "cocktail") dosing is one of the popular approaches for rapidly screening large number of compounds for their PK properties in vivo.[82] This approach has some implementation issues. One big concern is the drug–drug interaction in vivo following simultaneous administration of a number of structurally similar NCEs to a single animal. Due to their limited solubility in biological fluids, precipitation of the drugs in vivo would likely happen. In any of these situations, false positive or negative PK results remain a distinct possibility.[83,84] Therefore, managing the potential risk of missing a promising drug candidate is imperative.

Korfmacher et al.[5] introduced a rapid screening assay called cassette-accelerated rapid rat screen (CARRS) assay. In brief, one cassette contains six compounds and each compound is dosed in two rats and the plasma samples are collected at 0.5–6 h post-PO dose. The plasma samples from the two rats are pooled according to the collection time and assayed under a two-point linear calibration curve. The PK calculation is performed using an Excel-based template. This level I assay is designed to be easy to implement in a higher throughput manner. Table 3 lists in detail the rules for a level I assay.[1]

B. Level II: Lead Optimization Requirement

Level II assays are required for lead optimization studies (e.g., rat, dog or monkey IV/PO studies). The goal of this kind of study is to obtain

TABLE 3 Rules for Discovery (Non-GLP) "Screen" Assays (Level I)

1. Samples should be assayed using HPLC-MS/MS technology
2. Sample preparation should consist of protein precipitation using an appropriate internal standard (IS)
3. Samples should be assayed along with a standard curve in duplicate (at the beginning and end of the sample set)
4. The zero standard is prepared and assayed, but is not included in the calibration curve regression
5. Standard curve stock solutions are prepared after correcting the standard for the salt factor
6. The standard curve should be of three levels, typically ranging from 25 to 2500 ng/mL (they can be lower or higher as needed for the program); each standard is 10 times the one below (thus, a typical set would be 25, 250 and 2500 ng/mL). The matrix of the calibration curve should be from the same animal species and matrix type as the samples
7. QC samples are not used and the assay is not validated
8. After the assay, the proper standard curve range for the samples is selected; this must include only two concentrations in the range that covers the samples. A one order of magnitude range is preferred, but two orders of magnitude is acceptable, if needed to cover the samples
9. Once the range is selected, at least three of the four assayed standards in the range must be included in the regression analysis. Regression is performed using unweighted linear regression (not forced through zero)
10. All standards included in the regression set must be back calculated to within 27.5% of their nominal values
11. The LOQ may be set as either the lowest standard in the selected range or as 0.4 times the lowest standard in the selected range, but the LOQ must be greater than three times the mean value for the back-calculated value of the two zero standards
12. Samples below the LOQ are reported as zero.
13. If the LOQ is 0.4 times the lowest standard in the selected range, then samples with back-calculated values between the LOQ and the lowest standard in the selected range may be reported as their calculated value provided the S/N for the analyte to be at least 3
14. Samples with back-calculated values between 1.0 and 2.0 times the highest standard in the selected range are reportable by extending the calibration line up to two times the high standard
15. Samples found to have analyte concentrations more than two times the highest standard in the regression set are not reportable; these samples must be reassayed after dilution or along with a standard curve that has higher concentrations so that the sample is within two times the highest standard

Source: With permission from reference 1 (ISBN: 0-8493-1963-3).

enough data to be able to calculate PK parameters (e.g., clearance, volume of distribution, half-life, etc.). Therefore, these assays need to be more rigorous. Table 4 lists the more restricted rules in this level.[1] The key of being successful in this level of assay is getting a good regression based on a multiple-point standard curve. Typically, a triple quadrupole

TABLE 4 Rules for Discovery (Non-GLP) "Full PK" Assays (Level II)

1. Samples should be assayed using HPLC-MS/MS technology
2. Sample preparation should consist of protein precipitation using an appropriate internal standard (IS)
3. Samples should be assayed along with a standard curve in duplicate (at the beginning and end of the sample set)
4. The zero standard is prepared and assayed, but is not included in the calibration curve regression
5. Standard curve stock solutions are prepared after correcting the standard for the salt factor
6. The standard curve should be 10–15 levels, typically ranging from 1 to 5000 or 10,000 ng/mL (or higher as needed). The matrix of the calibration curve should be from the same animal species and matrix type as the samples
7. QC samples are not used
8. After the assay, the proper standard curve range for the samples is selected; this must include at least five (consecutive) concentrations
9. Once the range is selected, at least 75% of the assayed standards in the range must be included in the regression analysis
10. Regression can be performed using weighted or unweighted linear or smooth curve fitting (e.g., power curve or quadratic), but is not forced through zero
11. All standards included in the regression set must be back calculated to within 27.5% of their nominal values
12. The regression r^2 must be 0.94 or more.
13. The LOQ may be set as either the lowest standard in the selected range or as 0.4 times the lowest standard in the selected range, but the LOQ must be greater than three times the mean value for the back-calculated value of the two zero standards
14. Samples below the LOQ are reported as zero
15. If the LOQ is 0.4 times the lowest standard in the selected range, then samples with back-calculated values between the LOQ and the lowest standard in the selected range may be reported as their calculated value provided the S/N for the analyte to be at least 3
16. Samples with back-calculated values between 1.0 and 2.0 times the highest standard in the selected range are reportable by extending the calibration curve up to two times the high standard as long as the calibration curve regression was not performed using quadratic regression
17. Samples found to have analyte concentrations more than two times the highest standard in the regression set are not reportable; these samples must be reassayed after dilution or along with a standard curve that has higher concentrations so that the sample is within two times the highest standard
18. The assay is not validated
19. The final data does not need to have quality assurance (QA) approval. This is an exploratory, non-GLP study

Source: With permission from reference 1 (ISBN: 0-8493-1963-3).

TABLE 5 Additional Rules for Discovery (Non-GLP) PK Assays Requiring QC Samples (Level III)

1. Use all the rules for "full PK – level II" assays (except rule 7) plus the following rules
2. Quality control (QC) standards are required, and a minimum of six QCs at three concentrations (low, middle, high) are to be used. The QC standards should be frozen at the same freezer temperature as the samples to be assayed
3. The QC standards need to be traceable to a separate analyte weighing from the one used for the standard curve standards
4. The standard curve standards should be prepared on the same day the samples are prepared for assay – the standard curve solutions needed for this purpose may be stored in a refrigerator until needed for up to 6 months
5. At least two-third of the QC samples must be within 25% of their prepared (nominal) values
6. If dilution of one or more samples is required for this assay, then an additional QC at the higher level must be prepared, diluted and assayed along with the sample(s) needing dilution – this QC should be run in duplicate and at least one of the two assay results must meet the 25% criteria

Source: With permission from reference 1 (ISBN: 0-8493-1963-3).

mass spectrometer will give a linear response with three to four orders of magnitude in the dynamic range of the drug concentration. The regression weighing parameters are often $1/x$ or $1/x^2$, which is needed to make the low end of the standard curve fit correctly. At higher concentrations, the standard curve may deviate from a linear response due to ionization factors or instrumental detection limits. This non-linearity can often be compensated for by the use of either power curve or quadratic fitting when performing the regression step.[1]

C. Level III: Lead Qualification Requirement

When a drug candidate approaches development (e.g., enzyme induction study, single rising dose, radiolabel PK studies), an additional level of analytical rigor is needed for the assay. Since there are only a few compounds and studies that can reach this stage, data accuracy becomes a bigger concern. At this level, quality control (QC) samples are usually included to provide additional confidence for the results. Table 5 lists the additional criteria on the level III assay requirements.[1]

VIII. ASSAY SAMPLES AND GENERATE PHARMACOKINETIC REPORTS

As soon as the developed method meets all the criteria, the samples can be assayed and the PK parameters can be calculated and reported to drug discovery team. Simple PK calculations (e.g., AUC, C_{max}, T_{max}) can be performed using Excel or similar software. For more complicated PK

calculations (e.g., clearance, volume of distribution, mean residence time), a popular PK calculation tool is Watson LIMS™ (InnaPhase Corp.).[1] The Watson LIMS system is a highly specialized protocol-driven laboratory information management system (LIMS) specifically designed to support DMPK/bioanalytical studies in drug discovery and development. Watson uses a central Oracle database and offers a simple, point-and-click graphical interface. It is capable of handling standard and complex PK study protocols. It has full bi-directional interface capability to analytical instruments (e.g., LC-MS systems), supports a wide range of PK/TK analyses and generates study results in a unique document format.

IX. CONCLUSIONS

Revolutionary changes in mass spectrometry during the past decade, particularly the introduction of the robust, broadly applicable ionization techniques of ESI and APCI, have made mass spectrometry an essential tool in the optimization of the in vivo PK parameters of lead molecules in the drug discovery process. As both mass spectrometry and sample robotic instrumentation improve, there will continue to be opportunities for increasing the throughput of the discovery PK studies. The extensive discussion in this chapter has captured the most important elements that need to be addressed in method development. However, many of the choices and finer points of LC/MS method development will depend on the molecules to be determined.

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12

HPLC METHOD DEVELOPMENT IN EARLY PHASE PHARMACEUTICAL DEVELOPMENT

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ABSTRACT

The primary goal of early phase development is to gain a fundamental knowledge of the chemistry of drug substances and drug products to facilitate optimization of synthetic schemes and drug product formulations. At the same time, methods are required for release and stability studies to support clinical trials, and must assure that products are safe in vivo. Ultimately, the knowledge gained during early development translates into designing control methods for commercial supplies that assure patient safety and efficacy.

HPLC Method Development for Pharmaceuticals
S. Ahuja and H. Rasmussen, editors.

This chapter describes an iterative and systematic approach to HPLC method development, designed to meet the specified goals.

I. INTRODUCTION

During early phase pharmaceutical development, drug substance synthetic routes and drug product dosage forms are selected. This is typically an iterative process that from a chromatographic perspective requires HPLC methods to separate a potentially different set of impurities and degradation products as drug substance and drug product development advance. At the same time, methods are required for release and stability testing of clinical supplies to assure that the products are safe and effective in vivo. For this application, the methods need to unequivocally monitor all impurities and degradation products to assure that guidelines for reporting, identification and toxicological qualification[1,2] are met.

From these requirements, it is apparent that conventional method development schemes[3–8] that are based on optimizing the separation of a known set of analytes have limitations:

(1) The set of analytes to be separated is not known at the onset of method development.
(2) The set of analytes to be separated will change over time.

This chapter will endeavor to outline a strategy to accommodate these limitations. Sequentially the approach consists of

(1) Obtaining or generating, to the degree possible, samples of all of the impurities and degradation products that may be present in a drug substance or drug product.
(2) Developing an array of orthogonal HPLC methods that can collectively separate the active pharmaceutical ingredient (API) and all potential impurities and degradation products obtained or generated in (1).
(3) Development of a single primary method that can quantitate the API, and all potential impurities and degradation products assessed by the collective methods developed in (2).
(4) Use of the primary method and orthogonal methods for the analysis of new drug substance and drug product batches and critical drug substance and drug product stability samples. In this scheme, the primary method is used to quantitate the analytes observed to date. The orthogonal methods are used to assure that no previously un-encountered impurities or degradation products, not observed with the primary method, are formed. This step is used successively, until the final drug synthesis route is identified and the dosage form is finalized. Additional development may be

required on a case-by-case basis to address mass balance issues or to develop methods for genotoxic impurities.[9,10]

In parallel with, or following, these activities, the significant[1,2] impurities and degradation products are identified, drug chemistry is conducted to understand the origin of the impurities and degradation products, corrective action is implemented to minimize the formation of impurities and degradation products, and impurities and degradation products are isolated or synthesized. An ultimate goal of this approach is to provide a complete understanding of the impurities and degradation products that must be monitored in the final drug substance and drug product and to assure that authentic samples of these analytes are available for late phase HPLC method development.

In essence, early phase development activities are targeted towards full understanding of chemistry of the drug substance and drug product to remove the limitations previously cited as obstacles to implementation of conventional method development approaches.

II. FORCED DECOMPOSITION AND IMPURITY PROFILING

Prior to method development, a comprehensive assessment of the synthesis, and an evaluation of possible degradation pathways for the molecule should be conducted to obtain a preliminary indication of likely synthetic impurities and degradation products. It is beyond the scope of this chapter to provide a detailed discussion of synthesis and degradation pathways, but the importance of understanding the chemistry should certainly be emphasized. The interested reader is referred to a review article by Bakshi and Singh[9] for a more extensive discussion on degradation chemistry. A particularly important outcome of the assessment is to assure that potential impurities and degradation products that are extremely polar, extremely non-polar, have no or little UV activity or have potential genotoxic effects are postulated. These compounds may or may not be determined by the primary method development approach described in this chapter. Consequently, efforts should be directed toward assuring that the method is suitable for determining these compounds, if present, or alternatively toward developing additional methods to target these compounds specifically.

The actual method development process starts with obtaining or generating, to the degree possible, samples of all of the impurities and degradation products that may be present in a drug substance or drug product. This is generally accomplished by selecting a lot or lots of drug substance produced using different synthetic routes (as available), and generating potential degradation products via forced decomposition (a.k.a. forced degradation) studies. Guidelines and conditions

TABLE I Forced Degradation Stressing Conditions

Stressing condition	Temperature (°C)	Stressing time (days)
Water	70	0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 7
0.1N HCl	70	0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 7
0.1N NaOH	70	0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 7
0.3% H_2O_2	Ambient	0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 7, 10, 14
Water/cool white fluorescent light	Ambient	0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 7, 10, 14
Solid/cool white fluorescent light	Ambient	0, 1, 2, 3, 5, 7, 9, 10, 11, 12, 13, 14
Solid/suntest (UV and visible light)	Ambient	8 h (1×ICH), 16 h (2×ICH)
pH 2 buffer	70	0, 1, 3, 5, 7
pH 4 buffer	70	0, 1, 3, 5, 7
pH 6 buffer	70	0, 1, 3, 5, 7
pH 8 buffer	70	0, 1, 3, 5, 7
pH 10 buffer	70	0, 1, 3, 5, 7
Solid	Week 1: 60; week 2: 70; week 3: 80	0, 1, 3, 5, 7, 8, 10, 12, 14, 15, 17, 19, 21, 22, 24, 26, 28, 29, 30, 31
Solid/75% RH	Week 1: 60; week 2: 70; week 3: 80	0, 1, 3, 5, 7, 8, 10, 12, 14, 15, 17, 19, 21, 22, 24, 26, 28, 29, 30, 31

forced decomposition studies have been discussed elsewhere,[9,11] with numerous approaches being advocated. Our current approach is detailed in Table 1.

Samples are stressed using the conditions and times noted, and frozen immediately after stressing to quench the reactions. The samples can be prepared and pulled manually, or the process can be fully automated.[12,13] Solutions are typically prepared at 1 mg drug per mL of solvent, with the dilution step either preceding (for solution) or following (solid state) stressing. Upon completion of the study, the most stressed samples for each condition are analyzed using a single chromatographic method. This may be either a method established during drug discovery or a generic broad gradient method. If no degradation is observed for a condition, further studies on solutions obtained via the conditions are deferred for later analysis. If degradation above 15% is observed (by area %), less stressed samples obtained under the same conditions are evaluated to find samples with ~5–15% degradation. Solutions degraded above 15% have a larger risk of containing secondary degradation products that may not be

formed under less stringent degradation conditions. Accordingly, results for these samples may be misleading.

To illustrate, a drug substance was stressed as detailed above and analyzed using the following conditions:

Column: Zorbax Extend C18, 100 × 4.6 mm, 3.5 μm
Column temperature: 45°C
Sample temperature: 5°C
Detection: PDA – 280 nm (monitored at 190–400 nm)
Injection volume: 5 μL
Mobile phase: A=10 mM ammonium acetate; B=acetonitrile
Gradient:

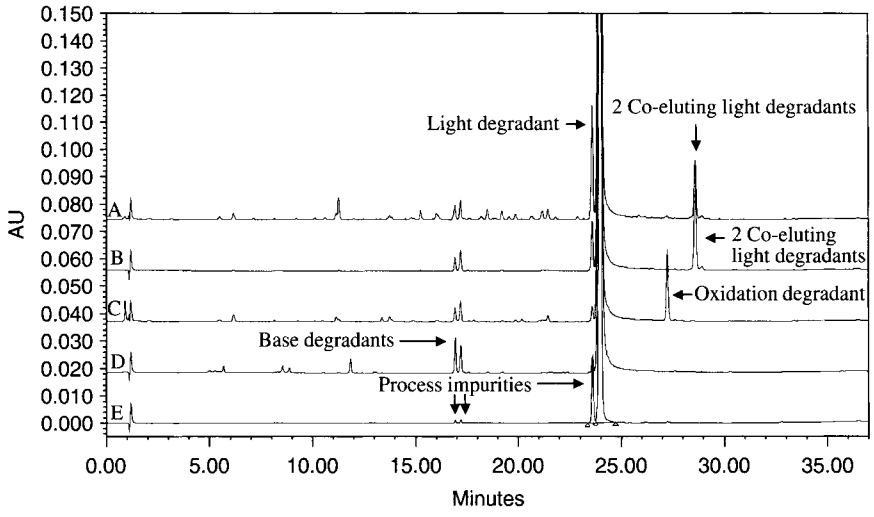
Time	Flow (mL/min)	%A	%B
0	1.0	95	5
30	1.0	40	60
35	1.0	10	90
40	1.0	10	90
42	1.0	95	5
50	1.0	95	5

The drug was found to be stable when stressed under acidic conditions (pH 2, 4, 6 and 0.1N HCl), basic conditions in buffer at moderate pH (pH 8, 10), heat and heat with elevated relative humidity (75% RH). However, degradation was observed when the drug was exposed to basic (0.1 N NaOH) and oxidative conditions (0.3% H_2O_2), and also when exposed to cool white fluorescent light (solid state and water) and intense UV and visible light (ICH option 1[14]). Chromatograms of the stressed samples and unstressed drug substance, containing synthetic impurities, are shown in Figure 1.

On the basis of this data, solutions B–D (Figure 1 legend) contain unique impurities and degradation products and were chosen for further method development using orthogonal screening.

III. ORTHOGONAL SCREENING

Method development using orthogonal screening is based on evaluating separation under conditions that provide different selectivity to maximize the probability that components that coelute under one set of conditions are separated using a different set of conditions.[15–17] Variation occurs from the use of different modes of HPLC, different stationary phases, mobile phase composition (organic modifier, pH, additives) and column temperature. Differences between columns and retention mechanisms have been discussed in Chapters 3 and 4.



A: drug substance in water stressed under fluorescent light for 7 days
B: solid stressed under fluorescent light for 7 days
C: drug substance stressed with 0.3% H_2O_2 for 7 days at Ambient
D: drug substance stressed with 0.1N NaOH for 7 days at 70°C
E: standard (0.175 mg/mL)

FIGURE 1 Chromatograms of significant forced degraded samples. (A) Drug substance in water stressed under fluorescent light for 7 days; (B) solid stressed under fluorescent light for 7 days; (C) drug substance stressed with 0.3% H_2O_2 for 7 days at ambient; (D) drug substance stressed with 0.1N NaOH for 7 days at 70°C; (E) standard (0.175 mg/mL).

Approaches to defining orthogonality have included the use of correlation coefficients from plots of retention factors,[18] or correlation coefficient color maps.[19] We have defined orthogonality as conditions under which the correlation coefficient from retention factor plots are less than 0.70.

Vastly different separations may also be achieved by using other separation methods with inherently different separation mechanisms. Capillary electrophoresis is potentially extremely attractive, since the inherent separation mechanism is different and since all components are eluted.[20] Hydrophilic interaction chromatography[21] has recently been demonstrated to provide excellent orthogonality,[22] and has the advantage over normal phase chromatography of allowing for the same (aqueous-based) samples to be used for evaluation.

From the perspective of designing a generic screening module for the purpose of assuring that all the methods will collectively allow for the determination of *all* impurities and degradation products, several practical criteria must also be considered:

1. The screening module should have broad applicability to a wide array of drugs.

2. Broad gradients (minimally 10–85% organic) should be used to maximize the probability that all analytes are retained and that all analytes elute from the column. For samples where extremely polar or non-polar impurities or degradation products are postulated, or to assure elution of all compounds within the gradient range, experiments beyond those described herein can be conducted following screening. Specifically, the applicability of the method to highly retained compounds (e.g. double-structures or oligomers) can be evaluated by eluting these compounds with organic solvents. This can be achieved via non-aqueous RP-chromatography. The applicability of the method to highly polar compounds can be evaluated by scanning the void volume region by LC-MS.[17]
3. Mobile phases should be compatible with MS to aid in peak tracking between experimental conditions and to allow for identification (where necessary).

For the study detailed in this chapter, the columns noted in Table 2 were selected. Refinement of this set is ongoing as additional experience is gained on orthogonality between the columns specified and as new columns with unique retention mechanisms are introduced. Avoiding conditions that are incompatible with the selected columns due to operating pH, each of the mobile phases specified in Table 3 is run using the generic conditions described in Table 4. All experiments are conducted

TABLE 2 Orthogonal Screening: Columns

Stationary phase	Column	pH range[a]	Manufacturer	Part number
C18 – Twin Technology	Gemini C18, 5 µm, 110A, 4.6 × 150 mm	1–12	Phenomenex	00F-4435-E0
Phenyl with hexyl (C6) linker, endcapped	Luna Phenyl-Hexyl, 3 µm, 4.6 × 150 mm	1.5–10	Phenomenex	00F-4256-E0
C18 – 20% C loading	Discovery HS-C18, 3 µm, 4.6 × 150 mm	2–8	Supelco	569252-U
C18 – polar embedded, hybrid particle with Shield Technology	XTerra RP18, 3.5 µm, 4.6 × 150 mm	1–12	Waters	186000442
C18 – silica	SunFire C18, 3.5 µm, 4.6 × 150 mm	2–8	Waters	186002554
Pentafluorophenyl	Curosil PFP, 3 µm, 4.6 × 150 mm	2–7.5	Phenomenex	00F-4122-E0

[a]Columns were screened only against mobiles phases within their compatible pH range.

TABLE 3 Orthogonal Screening: Mobile Phase Modifiers

Modifier	Mobile phase concentration	Approximate pH
Trifluoroacetic acid (TFA)	0.05%	2
Formic acid	0.1%	2.8
Ammonium acetate + acetic acid	8 mM + 0.1%	4
Ammonium acetate	8 mM	7
Ammonium acetate + ammonium hydroxide	8 mM + 0.05%	10.2
Ammonium hydroxide	0.05%	10.8

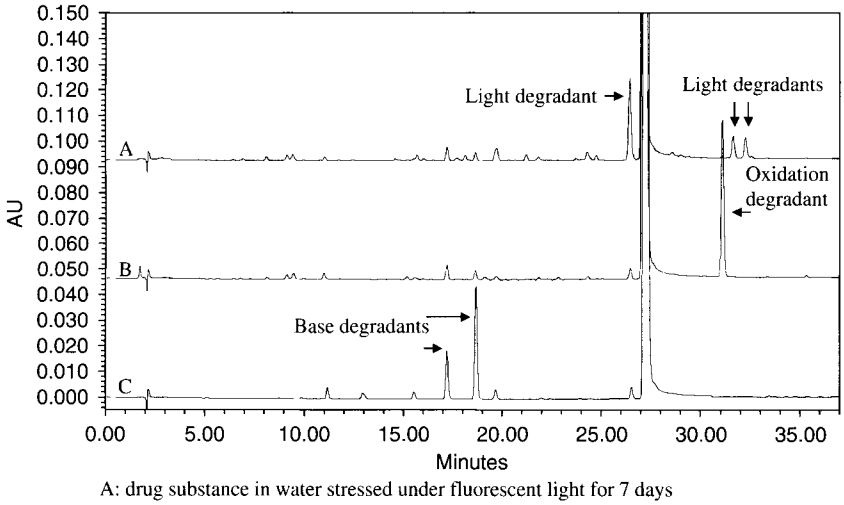
TABLE 4 Orthogonal Screening: Method Description

Time (min)	% Water	% Acetonitrile	% Modifier[a]	Flow rate (mL/min)
0	85	10	5	1.0
40	10	85	5	1.0
45	10	85	5	1.0
45.10	85	10	5	1.0
60	85	10	5	1.0
Injection volume		5 µL		
Detection		280 nm; DAD (190–400 nm)		
Column temperature		Ambient		
Sample temperature		5°C		

[a]Modifier stock solutions are prepared at a concentration 20 times higher than the desired mobile phase concentration since mobile phases are prepared at time of use with the HPLC quaternary pump.

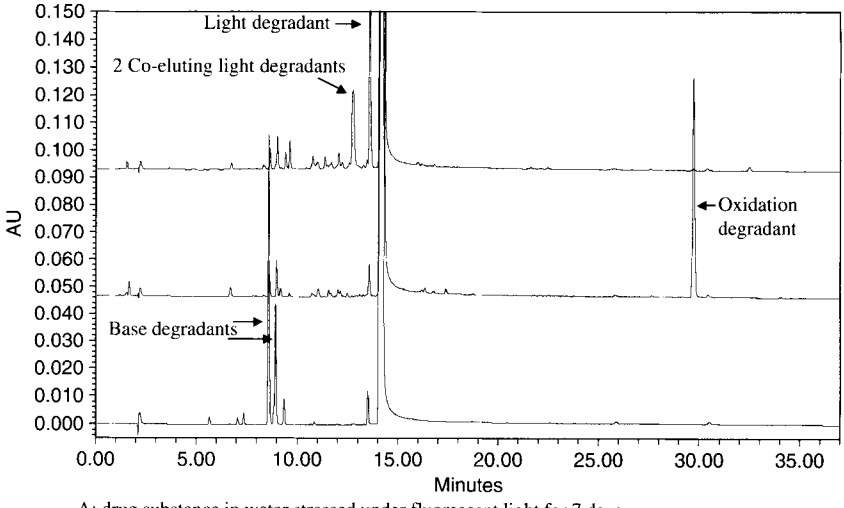
using a column-switching valve to allow for unattended operation. Full experiments conducted in this manner are typically achieved in approximately 96 h, depending on the number of individual impurity and degradation product samples analyzed under each condition.

Following completion of the experiment, the results obtained by each set of conditions are compared. As shown in Figures 2 and 3, very different elution orders are observed using an XTerra RP18 column with an acetate buffer and a SunFire C18 column with formic acid as the modifier. This is further demonstrated in Figure 4, from plots of retention factors for each peak observed on each of the two columns. Both conditions additionally provide fairly symmetrical peaks, and no other attributes that would be of chromatographic concern. From these data, the two separations are considered orthogonal and the two



A: drug substance in water stressed under fluorescent light for 7 days
B: drug substance stressed with 0.3% H_2O_2 for 5 days at Ambient
C: drug substance stressed with 0.1N NaOH for 5 days at 70°C

FIGURE 2 Orthogonal screening results: XTerra RP18 Column with 8 mM ammonium acetate + 0.1% acetic acid modifier. (A) Drug substance in water stressed under fluorescent light for 7 days; (B) drug substance stressed with 0.3% H_2O_2 for 5 days at ambient; (C) drug substance stressed with 0.1N NaOH for 5 days at 70°C.



A: drug substance in water stressed under fluorescent light for 7 days
B: drug substance stressed with 0.3% H_2O_2 for 5 days at Ambient
C: drug substance stressed with 0.1N NaOH for 5 days at 70°C

FIGURE 3 Orthogonal screening results: SunFire C18 Column with 0.1% formic acid modifier. (A) Drug substance in water stressed under fluorescent light for 7 days; (B) drug substance stressed with 0.3% H_2O_2 for 5 days at ambient; (C) drug substance stressed with 0.1N NaOH for 5 days at 70°C.

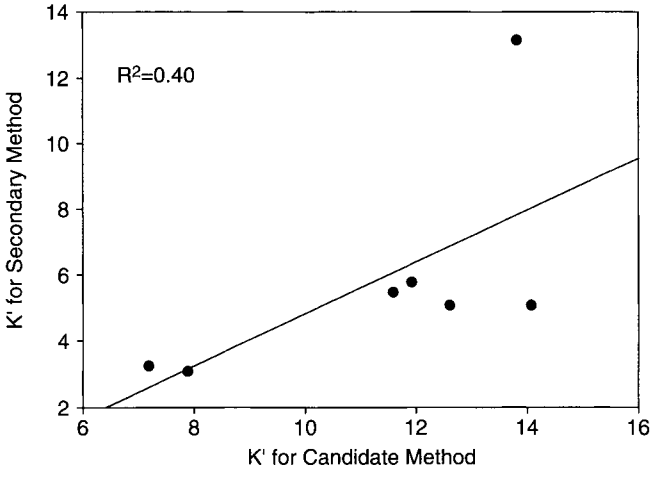


FIGURE 4 Orthogonality plot (pseudo-*K'* plot) for the candidate and secondary methods.

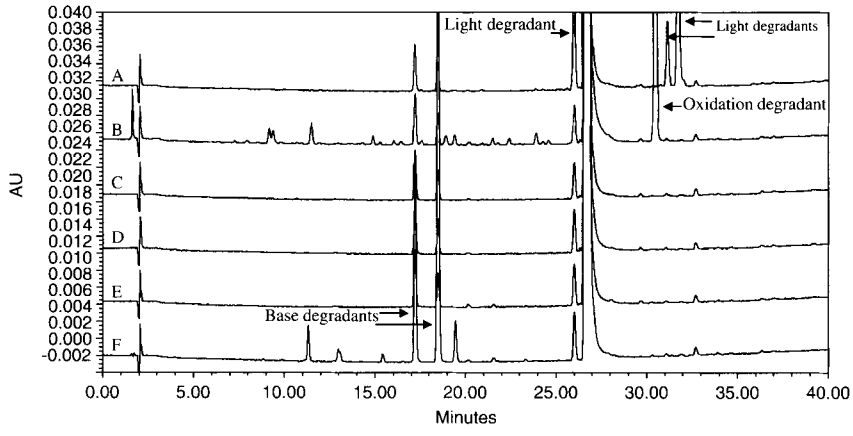
separations are tentatively considered as viable primary and orthogonal methods.

To further examine the two selected methods, the samples previously identified as containing degradation products, along with the most stressed samples obtained under other stress conditions are analyzed under both sets of conditions, to assure that no peaks were missed by the initial scouting run. Chromatograms obtained for both sets of conditions are shown in Figures 5 and 6, and demonstrate that no additional peaks are obtained under either set of conditions.

IV. METHOD SELECTION AND OPTIMIZATION

Following the completion of orthogonal screening, the most viable candidate method is optimized and validated and is used for release and stability testing. The orthogonal method is used to screen samples from new synthetic routes and pivotal stability samples of both drug substance and drug product. This ensures, as a first pass, that all peaks of interest are reported using the release method, and triggers the need for method redevelopment or use of additional control methods if additional, new peaks are observed with the orthogonal method.

It is important at this juncture not to "over-optimize" any method. While it is tempting to reduce analysis time by limiting the gradient range, it should be noted that new drug substance or drug product batches may contain new impurities or degradation products. Accordingly, optimized



A: solid stressed under intense UV & Visible light – 1× ICH
B: drug substance stressed with 0.3% H_2O_2 – 3 days
C: solid at elevated temperature (60°C) – 3 days
D: solidat elevated temperature and humidity (60°C/75% RH) – 3 days
E: drug substance stressed with 0.1 NHCl at 70°C – 3 days
F: drug substance stressed with 0.1 N NaOH at 70°C – 3 days

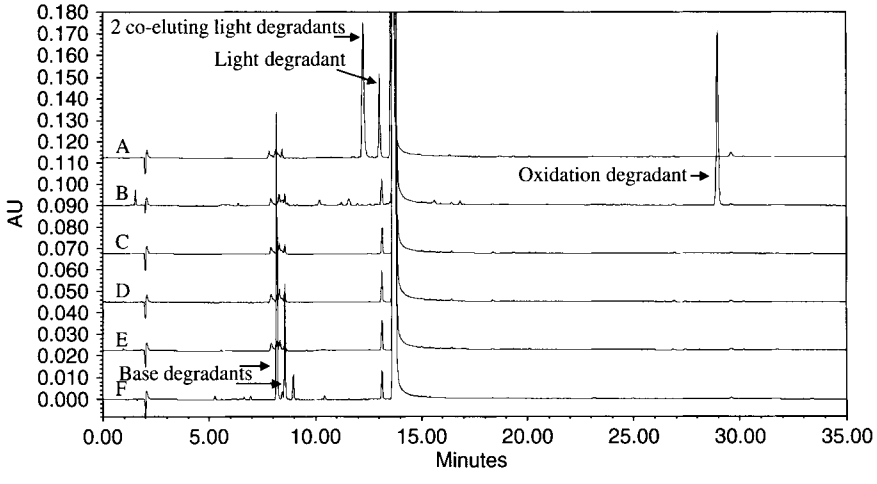
FIGURE 5 Candidate method: XTerra RP18 Column (4.6 × 150 mm) with mobile phase additive 8 mM ammonium acetate + 0.1% acetic acid – chromatograms of representative forced degraded samples. (A) Solid stressed under intense UV and visible light – 1 × ICH; (B) drug substance stressed with 0.3% H_2O_2 – 3 days; (C) solid at elevated temperature (60(C) – 3 days; (D) solid at elevated temperature and humidity (60(C/75% RH) – 3 days; (E) drug substance stressed with 0.1N HCl at 70°C – 3 days; (F) drug substance stressed with 0.1N NaOH at 70°C – 3 days.

methods may be short-lived. However, some optimization is warranted. Sequentially, the following activities are recommended:

A. Evaluation of Retention Time Reproducibility

Since different pH buffers were used during screening without regard for the pK_a(s) of the compounds separated, there is a possibility that the operating pH is close to the pK_a of a compound of interest. This can lead to large changes in retention times[17] with minor changes in buffer concentration and pH caused by the experimental error of replicate mobile phase preparations. As individual peaks are tracked using retention times or relative retention times (the retention time of the component divided by the retention time of the API) in subsequent analyses, retention time stability is a critical method attribute to prevent peak misidentification.

To study the ruggedness of the method with respect to pH, replicate buffers are prepared and the separation evaluated. The study may be combined with using two columns prepared from different batches of silica gel to simultaneously evaluate column influence. It should be noted, however, that column-related issues are greatly mitigated by choosing columns from a reliable source. Reproducibility of numerous columns



A: solid stressed under intense UV & Visible light – 1× ICH
B: drug substance stressed with 0.3% H_2O_2 – 3 days
C: solid at elevated temperature (60°C) – 3 days
D: solid at elevated temperature and humidity (60°C/75% RH) – 3 days
E: drug substance stressed with 0.1NHCl at 70°C – 3 days
F: drug substance stressed with 0.1N NaOH at 70°C – 3 days

FIGURE 6 Secondary method: SunFire C18 Column (4.6 × 150 mm) with mobile phase additive 0.1% formic acid – chromatograms of representative forced degraded samples. (A) Solid stressed under intense UV and visible light – 1 × ICH; (B) drug substance stressed with 0.3% H_2O_2 – 3 days; (C) solid at elevated temperature (60(C) – 3 days; (D) solid at elevated temperature and humidity (60(C/75% RH) – 3 days; (E) drug substance stressed with 0.1N HCl at 70°C – 3 days; (F) drug substance stressed with 0.1N NaOH at 70°C – 3 days.

has been systematically evaluated.[23–26] In cases where retention time shift is unacceptable, further method development is required.

B. Evaluation of Sensitivity

To be used effectively for release and stability and evaluation of the additional samples noted, the method must be capable of determining the API concentration and impurities and degradation products with a concentration of 0.05% (w/w) relative to the API.[1,2] Based on the capabilities of modern detectors, obtaining a linear dynamic range of 0.05–120% (linear dynamic range = 2400) is readily achieved by selection of appropriate sample dilution and injection volumes.

C. Evaluation of Solution Stability

An additional important experiment to be conducted to assure method viability is to critically examine the stability of both the API and impurities and degradation products in the selected sample solvent. It is

TABLE 5 Acceptance Criteria for Solution Stability

Sample	Relative difference between initial and value at time t
API	≤3.0%
Impurities	
Y = 1.0–2.0 times of RT	≤50%
Y = 2.1–10 times of RT	≤30%
Y = 11–20 times of RT	≤20%
Y = 21–100 times of RT	≤10%
New degradation products	≤RT

generally desirable to have solution stability for up to 7 days to assure that samples can be re-analyzed as a first recourse in an out-of-specification (OOS) investigation.

Typical requirements for solution stability are provided in Table 5. Generally, samples should assay within 3% during the time period over which solution stability is mandated. For impurities and degradation products, less stringent criteria are applied, and are linked to the level at which the impurities are observed, relative to the reporting threshold. It is additionally desirable that no new degradation products above the reporting threshold are formed.

Important experimental data for selection of the sample solvent can be obtained from the orthogonal screening study conducted on the degraded samples. If, for example, the analyte is shown to degrade in basic media, an acidified sample solvent may be preferred. Likewise, if the samples show light degradation, precautions should be taken to use appropriate glassware and/or room lighting. At times prevention of sample degradation may require for samples to be stored at sub-ambient temperatures. While this should be avoided, if not necessary, the need for 7-day sample stability does justify placing criteria on storage conditions.

D. Considerations for Drug Products

For drug products, the selection of sample solvent is further complicated by adding the additional criterion that a quantitative transfer is required from the sample matrix to the sample solvent. Sample preparation is discussed in detail in Chapter 8, and a comprehensive discussion will not be repeated in this chapter. However, it is noteworthy to mention that the sample preparation procedure should be re-examined when the sample is changed. Changes may include not only compositional changes to the drug product but also changes in manufacture, such as changes in tableting compaction force, etc.

When formulation changes include a change in excipients, it is also worthwhile to repeat the light, temperature and humidity forced decomposition studies to assure that no new degradation products are formed. New degradation products may result from interaction with the excipients, with interactions with impurities in the excipients or via catalysis from residual metals in the excipients.

V. ADDITIONAL METHODS

Following completion of the activities detailed, HPLC methods are now available to allow for the determination of API, impurities and degradation products in both drug substance and drug product. For use in release testing and stability studies, the primary method is validated.[27] Validation is discussed further in Chapter 16. In general, less extensive validation is required for early development methods than for methods intended to support release of commercial supplies.[17,28] The methods are used, as discussed previously to examine successive lots of drug substance and drug product, including stability samples, to obtain a chemical understanding of the impact of synthetic changes and formulation.

An important attribute of the methods is the use of MS compatible buffers. This facilitates direct use of the methods for HPLC/MS, and thereby structural elucidation. However, in some instances conditions will need to be modified to optimize MS performance. Other techniques such as HPLC/NMR (see Chapter 7) may similarly be used for determination of structures, and will frequently require separate or modified methods. Chemical syntheses or preparative separations are additionally possible to answer specific questions. Generally, such procedures have to be conducted to obtain pure reference materials for impurities and degradation products prior to final method development (see Section VI). Accordingly, the payback for generating samples of authentic substances is potentially larger than only to answer questions related to drug chemistry, especially if the impurity or degradation product cannot be eliminated by synthesis or formulation activities, and needs to be specified in control methods for commercial supplies.

In some instances, the methods may also not be able to sufficiently measure all potential impurities and degradation products. Examples include methods for potentially genotoxic impurities and impurities with limited UV response. For potentially genotoxic impurities, low ppm (relative to API) levels must be monitored.[10] Both the linear dynamic range of a UV detector and the consideration that the sample may contain numerous additional impurities at the ppm level, make the primary method unsuitable for genotoxic impurities. Generally, HPLC with more specific detection such as MS is required, although other separation techniques such as GC have also shown applicability.[29]

Strategically, it is important to develop a method for genotoxic impurities, where suspected, as early as possible in the development process. A goal should be to refine synthetic routes to eliminate the formation of these impurities. As such the method needs to be capable of clearly demonstrating the absence of such compounds. Early development of the method allows for profiling of a sufficient number of batches to demonstrate the absence of the potential genotoxic impurity, and will hopefully result in eliminating the need for the method as a control method for commercial supplies.

Additional methods are also required for impurities that do not have an appreciable UV chromophore. Successful postulation of synthesis and degradation pathways will allow for the early determination of the need for such additional methods. Further need may be demonstrated if mass balance is not observed (see Section VI). Compounds without chromophores are generally detected using evaporative light scattering detectors (ELSDs) or, more recently, nitrogen chemiluminescence detectors (NCDs).[30,31] ELSDs provide (near) equimolar responses to analytes, and NCDs provide (near) equivalent responses to analytes containing nitrogen. Accordingly, the detectors may be used to detect components that were previously unaccounted for as a result of poor molar extinction coefficients, and provide a reasonable quantitative estimate of impurities based on comparison to impurities of known concentration.

VI. SETTING THE STAGE FOR LATE PHASE HPLC METHOD DEVELOPMENTS

Following finalization of synthetic routes and drug product formulation(s), a comprehensive batch overview of the results generated by each method developed should be conducted and the following questions addressed:

(i) What related substances have been observed at a level above the reporting threshold in drug substance and drug product?
(ii) Are the related substances synthetic impurities, degradation products or both?
(iii) Have the impurities or degradation products observed been reduced to levels below the reporting threshold by changes in synthesis or drug product formulation?

With these questions addressed, design criteria can be generated for the drug substance and drug product method. Specific levels of impurities and degradation products, with no atypical toxicity, that need to be monitored are based on the drug's daily dose.[1,2] For typical dosages, drug substance and drug product methods must be capable of the following:

For drug substance: Methods should separate the API, synthetic process impurities and drug substance degradation products. Methods should

be able to detect impurities and degradation products present at levels of greater than 0.05% relative to the API.
For drug products: Methods should separate the API and drug product degradation products from excipients. Drug product methods are not required to monitor synthetic process impurities, unless they are also drug product degradation product. Methods should be able to detect degradation products present at levels of greater than 0.1% relative to the API.

Thus, while it is desirable to maintain the same methods for drug substance and drug product during early development, the focus of final methods are different and accordingly separate methods may be developed. It is important to conduct this activity as significant time saving may be achieved by developing new methods rather than continuing the use of the existing methods. Since the compounds to be separated are now known, conventional method development strategies[3–8] may be utilized. The original methods developed should, however, continue to be used for troubleshooting and should be transferred (where applicable) to laboratories charged with conducting analyses of commercial supplies. The development of final methods is discussed further in Chapter 13.

To complete the early development activities, two additional activities should also be conducted. The relevant (specified) impurities and degradation products not previously synthesized or isolated, should be generated and mass balance studies should be conducted to validate that the early development methods provide adequate control for all relevant impurities and degradation products.

Synthesized or isolated impurities and degradation products should be fully characterized with respect to both identity and purity. A purity assessment is important to allow for the establishment of relative response factors (the area/weight response of the impurity or degradation product relative to the area/weight response of the API). This allows for subsequent studies to use an API reference standard for quantitative analysis of both the API and the impurities and degradation products. A separate determination of response factors is required when changes are made to the method.

During early development it is also critical to examine mass balance for the separation, i.e., to establish that the summation of API, impurities and degradation products =100% on an anhydrous basis (within experimental error) in stressed samples. Key concerns typically associated with achieving mass balance include:[9]

(1) Degradation of the API to a volatile component.
(2) Diffusive losses into or through containers.
(3) Elution or resolution problems.
(4) Use of inappropriate response factors for quantitation of impurities/degradation products.

The method development strategy proposed here is designed to largely eliminate issues related to (3) and (4). Factors (1) and (2) are highly dependent on the specific nature of the problem, and may have to be considered on a case-by-case basis.

VII. CONCLUSIONS

HPLC methods are necessary to support clinical release and stability activities and evolutionary synthesis and formulation optimization. To address changes in the impurities and degradation product profiles generated during these activities, a systematic approach to method development, using an array of methods, is advocated as a means of obtaining full knowledge of drug substance and drug product chemistry. The approach places heavy emphasis on the use of orthogonal screening as a central activity to gather the knowledge required as a pre-requisite to developing methods suitable for the control of commercial supplies of drug substance and drug product. Key elements of the approach include the generation of degradation products via forced decomposition, and a continual evaluation of samples generated during the early development cycle.

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HPLC METHOD DEVELOPMENT IN LATE PHASE PHARMACEUTICAL DEVELOPMENT

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ABSTRACT

Method development is a process in which both the development lab and the receiving lab have to work closely in order to generate a test method description that is well documented, easily understood, and simply performed by an appropriately trained analyst.

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Within a quality control (QC) environment it is not important to have scientifically the most fancy method, but a technically straightforward, ready-to-use and most robust, durable, and good manufacturing practice (GMP) -compliant method is preferred. After a successful development, validation, and transfer, the method development lab should still be kept accountable for the performance of the method. Tracking of the output is key to understand where gaps are situated. Additionally, discussions on method shortcomings are then based on facts and figures, allowing for a better partnership between the development and the receiving labs. Understanding gaps results in learning of the method development process that in turn is favorable for improvement in future method development endeavors.

I. INTRODUCTION

Late phase methods are developed for registration stability studies and for release of the drug product (DP) or drug substance (DS) validation batches. Additionally, the methods are intended for transferral to the operational quality control (QC) laboratories for release testing of production batches. In the ideal case, the analytical methods should last for the entire product lifetime. Therefore, the aim of late phase method development is to develop fast, robust, reliable, and transferable high-performance liquid chromatography (HPLC) methods. In this context, it is crucial to devote adequate time, thoughts, and resources to the development of late phase analytical methods. Precise, accurate, robust, reliable, and transferable methods are required for QC testing.[1]

The classical approach of method development in many pharmaceutical companies usually is that the analytical development department develops the method and then transfers it to the QC or the stability lab. In most cases, the development group tries to complete its task as well as possible by developing the method to their best effort. The method is validated according to standard operating procedures and transferred to the application labs. The transfer exercise is considered to be the real challenge of the method. Both the development and the application labs are usually very excited and anxious to know the outcome of the transfer activity. However, many method-related surprises are frequently observed at this stage and have to be resolved if possible. In cases with more severe issues, the transfer fails and the method has to be redeveloped. The resulting delay may heavily impact the product development timelines and even jeopardize the filing date.

In the classical approach, the development group is usually focused fully on the task of method development and as a result they merely have the opportunity to talk to the application labs (sometimes the potential QC labs are not even known). The application labs on their side are also focused on doing their task and talking to the development lab may even be considered a waste of time. As a result, the needs of the customers are not accounted for during method development, which may potentially give issues during transfer and even complaints during

application. Additionally, the performance of the method during real analysis at the customer site is not known to the development lab and, therefore, complaints are generally perceived as subjective and not real.

II. GOALS OF DEVELOPMENT

In an extensive investigation of out of specification (OOS) results from different stability evaluation programs of different drugs, it was determined that analytical methods account for approximately 8% of the observed OOS results. Therefore, the analytical method development process was identified as an area for quality improvement in terms of robustness, reliability, method capability, and customer focus. HPLC methods were of particular concern due to their broad use in assaying the active pharmaceutical ingredient (API) and related impurities. To address performance concerns, a Six Sigma road map was followed to troubleshoot and improve the existing method development process. In this way (i) the voice of the customer was captured, (ii) key process input variables were identified, (iii) critical-to-quality (CTQ) factors were determined, (iv) several method verification tests were designed, (v) proactive evaluation of method performance during development was established, (vi) continuous customer involvement and focus was institutionalized, and (vii) method capability assessment (suitability to be applied for release testing against specification limits) was introduced.

An excellence approach in method development aims for a "right the first time" development process of late phase methods, with customer focus and through robust method development. Robust, reliable, and transferable methods result in reduced customer complaints, less rework, improved quality of the methods, monitoring of method performance, improved partnership with the customers, significantly reduced OOS, and a high probability for success during method transfer. Therefore, a redesigned approach is needed that starts with the generation of a method definition requirement document (MDRD). In the MDRD target, values are set for many CTQs prior to the start of method development. During the course of the method development process, design of experiment (DOE) approaches and measurement system analyses (MSA)[2] studies are systematically performed. The performance of the process is continuously monitored by a formal feedback loop. A schematic representation of the process is depicted in Figure 1. As can be observed there are no departments included in such an approach. The method development process is a continuous process in which all stakeholders are involved. The goal is to improve method quality first of all and then to reduce the complaints by the customers. This is achieved by involvement of the application labs right from the beginning of method development by partnering with them. The customer's voice is captured and accounted for during method development. General needs are translated

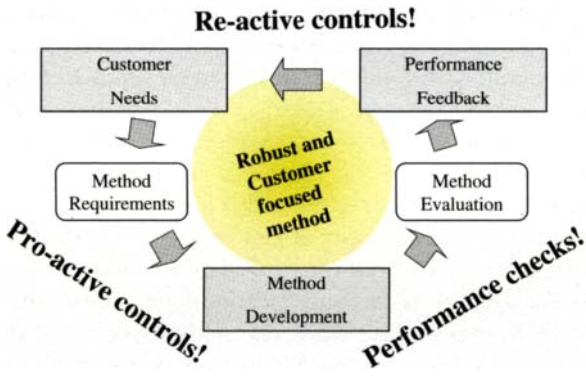

FIGURE 1 **Schematic overview of a method development process. Method development is a continuous process in which all stakeholders have to collaborate intensively to design the final method. The customer needs are translated into requirements that are considered during development. Developed methods are challenged extensively, by the development and the application labs, by method evaluation tests prior to full validation. Method performance is monitored and communicated to all the stakeholders. The data are used to discuss issues related to the method based on objective data.**

into critical-to-quality parameters and treated as such during development. Product specific customer needs are captured in the method definition document, a process in which all stakeholders are asked to participate.

The HPLC method can now be developed by taking into consideration all requirements as described in the MDRD and general chromatographic criteria as described in the previous section. Method optimization can be performed by either an intuitive, experimental design, or computer-assisted approach, and should deliver an optimized separation of the main compound and all relevant impurities in a reasonable analysis time using typical HPLC conditions. This is an activity that is mainly performed by the development group. The developed method should be evaluated for robustness and daily performance from lab to lab. Can the method be implemented in all the application labs? Is the method description acceptable? Are all customer needs accounted for? Again the customers play an important role in most of these activities.

Following method evaluation and the finishing optimization, the final method description is drafted, fully validated, and transferred to the application labs. After a thorough evaluation, these activities are expected to be carried out seamlessly. Method transfer *has* to be successful since by then the application labs already know the method because of their close involvement during development. Performance of the method is monitored each time samples are analyzed by the application labs. The gathered information is provided as method feedback to the development lab so that they are continuously informed about both the good and poor

method performance. Potential issues are discussed based on objective data and are resolved in close collaboration with each other. As can be derived from Figure 1, the method development approach has proactive controls (method requirement), performance checks (method evaluation) and reactive controls built within the process, to reduce customer complaints, avoid rework, improve method quality, track method performance, involve the customers, and promote partnership.

As mentioned before, late phase methods are developed in close collaboration with the customers (QC labs) and it is even highly recommended to involve the customer right from the beginning. The involvement of the customer is clearly demonstrated in the diagram, presented in Figure 2, which describes the late phase development process in more detail. The collaboration of the development lab (supplier) and the receiving lab (customer) should be like two pieces of a puzzle that closely fit each other to complete the overall picture. In a joint effort, both parties work together to develop their product: i.e. the analytical method. In this way, the quality of the delivered method is guaranteed. The late phase development process is performed in different steps and generally takes a couple of months to get completed. The following steps are included:[3] planning, method development, method evaluation, method validation, method transfer, and method performance monitoring.

III. PLANNING PHASE

A. Method Definition

Before starting method development, the requirements for the late phase method are set in an MDRD. The MDRD is generated in close collaboration and in agreement with all stakeholders, such as quality assurance (QA), QC sourcing sites, stability, development, and regulatory. It is a formal document in which all parties involved agree upon the method development requirements. The development process starts with a thorough planning step in which a development plan is generated based on the method requirement definition, available information gathering, and resource planning. The method development plan is generated in close collaboration with the customers. All stakeholders in the method define requirements for the method to be developed based on the existing knowledge. Timelines are set and a communication plan is made. The same method development team will evaluate the delivered method for attainment of the set requirements. In order to allow a smooth development process of the late phase methods, a couple of prerequisites are preferentially fulfilled:

- The synthesis route is locked and all critical intermediates and starting materials are defined for a DS method.

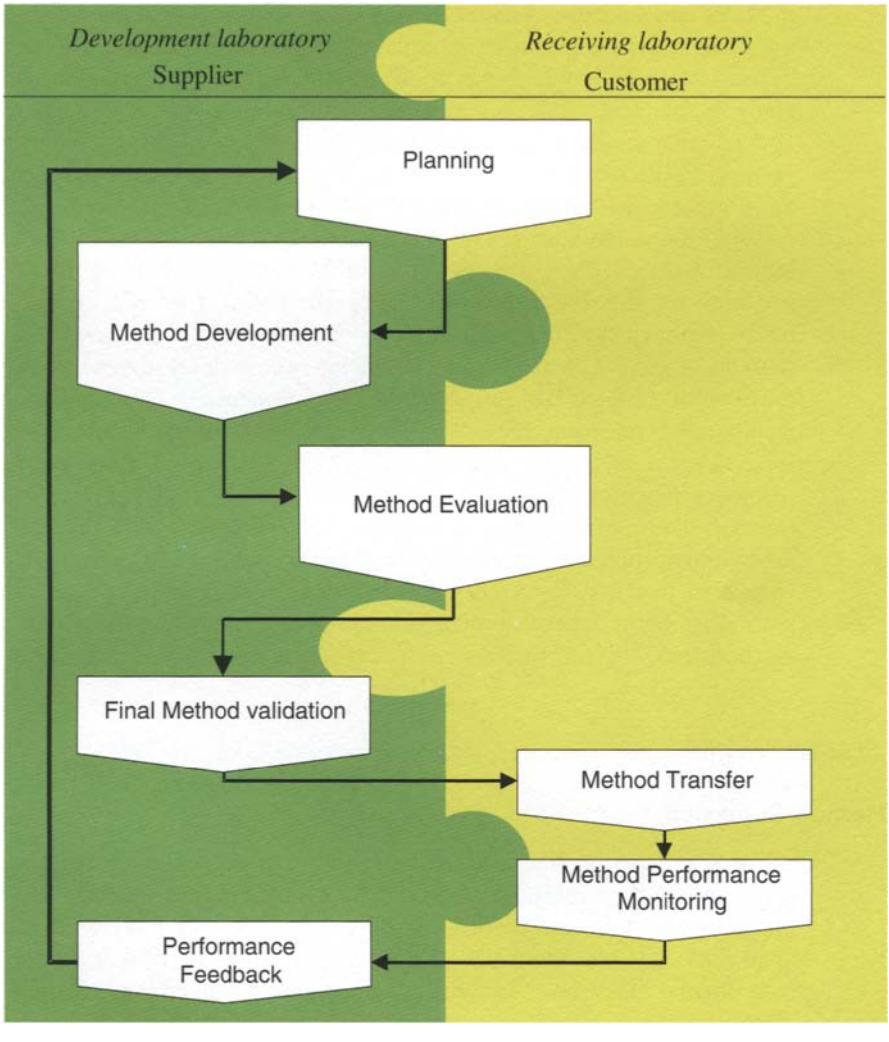

FIGURE 2 **Diagram illustrating the full method development process for late phase methods.**

- Formulation and dosage form compositions are locked for a DP method.
- Relevant impurities and degradation products are known and available as certified standards.
- Certified standard of the main compound and a selectivity batch or selectivity samples are available.
- Late phase product specifications are set in draft.

- Method development samples (appropriate DS batches, formulations (API and placebo)) and stability samples are available.

B. Helpful Information

During method development, information on the product is very helpful and sometimes even crucial. Therefore, it is recommended to gather all the information that is available from the early development period. The following information may be useful:

- UV spectra, solubility, pKa, stability of API, and model compounds.
- Early methods for characterizing DP and DS.
- Method development history.
- Late phase formulation composition.
- Late phase synthetic route.
- Stability data for DS and DP.
- Safety data (crucial).
- Excipient compatibility data.
- Information in regulatory files (e.g. clinical trial applications).
- Review of existing literature and current compliance guidelines and procedures.
- Feedback on early phase methods.

C. Development Plan

A development plan taking into consideration the previously mentioned requirements, together with an acceptable timeline is generated. This plan is communicated to all the stakeholders and major issues are discussed regularly. In addition to timelines, requirements for laboratory staff, instrumentation and equipment, materials/consumables, development samples, and standards are determined.

D. Method Requirements

1. General Requirements

Late phase methods are considered to deliver product characterization data of both the DS and DP. There may be many considerations that are targeted to meet the criteria for late phase methods. It is useful to have the same chromatographic system for the DS and DP method to allow for a comparison of impurity profiles. However, differentiation of

DS and DP methods should be allowed in order to meet specific and general objectives/requirements for the products. Assay of main compound and relevant related compounds is recommended to be performed in one chromatographic run. For DP, it is not necessary to quantify synthetic impurities, but synthetic impurities are labeled when they occur in the chromatogram. Although the guidelines[4,5] generally require reporting of unknown impurities above 0.10% in DS and 0.20% in DP, it is recommended to develop methods that are able to detect impurities at low levels. The target value of the reporting threshold should therefore preferentially be >0.05% w/w (or higher, depending on the product characteristics) in the purity methods.

Since lab cycle time is very important it should be aimed to develop methods with short run-times. Typical HPLC run-times of 30 min are considered to be acceptable. The compounds to be separated should be selected by the development team with all the method stakeholders. For precise and robust quantitative analyses, the separation of the peaks of interest should have target resolutions of >2.0, but sometimes resolution values of 1.5 may be sufficient. Methods for different strengths of similar formulations of the same API are preferably developed in parallel and should be more or less comparable. In such a way, QC labs do not have to change method conditions drastically from one analysis to the other, which certainly impacts analysis throughput time, user-friendliness, and the overall quality of the work. Similarly, the methods for different DS qualities are preferably the same.

2. Technical Requirements

In order to avoid out-of-linearity issues of the detector response, the maximal detector response for the nominal concentration of the API (100%, w/w) should be about 75% of the qualified linear dynamic range of the detector. Late phase methods are developed at temperature controlled conditions. Preferably around 35°C, since this is slightly higher than ambient temperature and can be achieved by ordinary HPLC column ovens. Mass spectrometry (MS) compatibility of the mobile phase is not required, but preferred. When non-volatile buffer systems are used in the mobile phase, an alternative MS compatible supportive method should be made available to support identification purposes. Buffer systems in the mobile phase are used when pH is found to be critical in robustness studies and are prepared by accurately weighing or pipetting of the two components of the buffer system. Adjusting the pH during buffer preparation should be avoided since this can cause poor robustness. It is, however, recommended to check the pH after preparation of the buffer solution. Tetrahydrofurane (THF) in the mobile phase is avoided where possible and is not used in preparation of the sample solution. The maximum concentration of the buffers should not be more than 40 mM because of the risk of precipitation during mixing with

organic solvents in a gradient run. Buffer solutions with pH values above the specified limits of the column are avoided because of the risk of dissolution of the silica gel.

When feasible, isocratic elution mode conditions are preferred for late phase methods because of better reproducibility. When isocratic conditions cannot be used, linear gradients are preferred. Step gradients may be used for the reduction of analysis time if there is a late eluting compound with a high capacity factor. After the last eluting peak, the run is preferentially continued for a couple of minutes in order to detect potential impurities or degradation products. The initial percentage of organic modifier in the mobile phase is preferably not less than 5%, which will allow better mixing properties in the mobile phase and avoid precipitation issues. It is also a good practice to continue the gradient up to a maximum of 95% organic modifier, when feasible and compatible with the salt type and concentration. Premixing of the mobile phase is preferred for robust performance. For example, when in a gradient elution method, two solvents A and B are applied in the mobile phase and the gradient program is such that it starts at 30% B and ends at 60% B, a (pre-)mixture of solvents A(70%) and B(30%) is prepared and hooked in line 1 of the HPLC pump. Another (pre-)mixture of solvents A(40%) and B(60%) is prepared and hooked in line 2 of the HPLC pump. The gradient will now be programmed to start at 100% solvent in line 1 and end with 100% solvent in line 2. In this way, mixing incompatibilities are avoided during the HPLC run. The inclusion of a rinsing step at the end of the gradient should always be considered, especially for DP analysis, where placebo ingredients may retain the stationary phase and potentially result in ghost peaks when eluted from the column. The mobile phase composition at starting conditions of the gradient is preferably also used as sample dilution solvent. Differences in solvent strengths are usually the cause of peak distortion (splitting) in the early part of the chromatogram.

A suitable, globally available HPLC column is used in accordance with manufacturer's specifications. For example, some columns are not to be used above pH 7 because of silica degradation. Other columns are specifically designed to be used at high-pH conditions. Column durability should be tested using the specified test conditions. An alternative column to be used in the method should be considered and appropriately validated. The initial pressure for a typical new column of 15-cm length and 5-μm particles, using typical HPLC conditions like a flow rate of 0.5–1.0 ml and ordinary buffer systems should be <200 bar.

Sample preparation is a crucial step in the analysis. First of all the sampling itself is very important. A typical sample preparation scheme consists of a sampling step, a weighing into suitable glassware, a disintegration/milling step, an extraction step that is usually facilitated by shaking, stirring, etc., a dilution, and a filtration or centrifugation step. For reasons of poor reproducibility, small volume pipettes are avoided

and volumetric flasks between 25–500 ml volumes are used. The weighed reference standard amounts for DS and DP are preferentially >100 mg (for DP amounts of >50 mg are acceptable)[6] for better precision. At least 10 sample solid units are used in the preparation of the sample solutions for DP. Alternatively, the sample may be prepared from a ground composite of at least 20 solid units. However, the general tendency is to avoid grinding since this is tedious and not user-friendly. The number of manipulation steps in the sample preparation protocol should be minimal since the analysts in the testing labs will have to do the work (hopefully for the long period of time that the product is on the market). Dilutions should be made directly from initial stock solutions with the lowest number of dilution steps.

IV. METHOD DEVELOPMENT PHASE

Late phase method development is started with a review of the available methods from early development. The method is evaluated against the method requirements set in the MDRD. If necessary, the method is optimized or re-developed in order to fulfill the requirements. Sometimes it may be needed to start development from scratch including method selection, definition of the parameter space and initial experiments. The next steps include the selectivity and system optimization, followed by method validation.[7] Different approaches can be applied to select the initial conditions.[8,9] Screening experiments as a function of the pH and different HPLC column stationary phases, as described earlier in Chapter 12, are applied. The outcome of this exercise is a first draft method description that should be challenged with selectivity (stressed) samples to evaluate its appropriateness. It may be required that the method has to be optimized further. Additionally, customers or experts in the field may be requested to review the draft method description.

A. Approaches to Method Optimization

1. Intuitive Approach

A method can be optimized intuitively based on the knowledge of an experienced HPLC expert. Indeed, the practical expertise of local staff can be of great value in resolving many typical chromatographic issues. However, recent challenges in the pharmaceutical field include analysis of a very broad variety of chemical compounds, ranging from small molecules of different types, through peptides to proteins. Moreover, due to the complicated chemical synthesis, the similarity and the number of related compounds to be separated from the main compounds may be high.

Additionally, the regulatory authorities demand to detect these impurities down to very challenging low levels.[4,5] In pharmaceutical companies, there are many chromatographic specialists available, but all the cited challenges make life very difficult even for the most experienced expert. As a result, the predictability of the intuitive approach is poor and may not always result in a consistent quality from analyst to analyst or from lab to lab.

2. Experimental Design Approach

By applying experimental designs, several variables can be investigated simultaneously. An experimental design is a "*planned series of operations called experiments in which certain conditions are held as constant as possible throughout and others are varied under the close control of the experimenter*".[10–12] After having selected the important variables for selectivity, the experimental domain has to be defined and mapped. The knowledge previously obtained during the selection of the important variables is used to define the experimental domain. As the parameter space cannot be expanded in an unlimited fashion,[9] it is essential to include only the variables that have the highest effect on the selectivity and to do this in appropriate level ranges. Within the experimental domain the migration of the compounds must be feasible, i.e. a peak of each compound with an acceptable shape and analysis time must be observed when the compound is injected under the conditions specified in the experimental domain. The feasible experimental region is determined by examining the migration of the compounds at the expected boundaries of the region.[13,14]

The selection of the experimental design is based on the feasible region and the migration behavior. In Figure 3, a decision tree is presented that can be used to select a design. When the feasible region is regular, a conventional experimental design is applied. The expected behavior of the migration characteristics can be linear straight line or curvilinear. A linear straight line migration behavior in a regular region may be examined by two-level factorial designs, whereas a curvilinear migration behavior should be examined by more level factorial designs, such as the star, central composite (which is a combination of a star and a full factorial design), Doehlert, the face-centered cube designs, etc. When the feasible region is irregularly shaped, there are two possibilities. One can try to reduce the region and fit a conventional design within it, although this limits the possibility of finding a good optimum (as the feasible region is decreased in size), or apply an advanced design. The more advanced experimental designs are not fixed to a geometrical shape, but are defined depending on the practical situation by certain algorithms. Examples of such designs are the Kennard and Stone or D-optimal designs.[15,16]

In factorial designs, the variables are called factors. The factors are investigated at two or three levels, denoted as −, 0, and + for "lower," "nominal," and "upper" levels, respectively. When there are only two

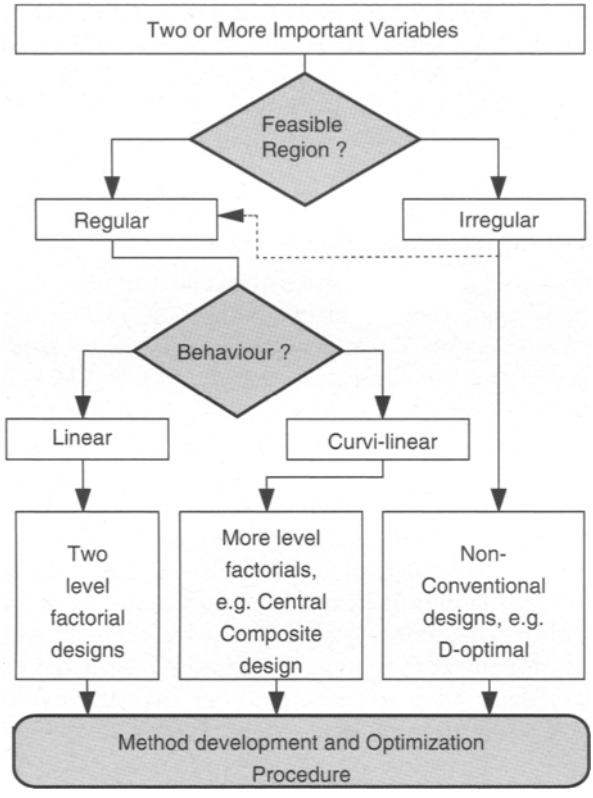

FIGURE 3 **Decision tree for the selection of an experimental design.**

levels, e.g. upper and lower levels, then such a design is called a two-level factorial design. For example, a two-level design of three variables results in 2^3 runs and is called a 2^3 factorial design. The design layout or "matrix" for a typical two-level full factorial design of three factors (a 2^3 factorial design): the pH, the temperature, and percentage of organic modifier are presented in Table 1. Additionally, Figure 4 gives a geometric representation of such an experimental design.

The influence of a factor is expressed by its factorial effect or main effect on the (quantitatively) measured response variables (y). Factorial effects are calculated for the factors as such and for possible interactions. The significance of the obtained factor effects is determined in several ways. A fast and clear approach is the graphical method where the obtained effects are included in a normal probability plot.[10,11,17,18] Another way of evaluating the significance is by a t-test in which the factor effect is compared with the experimental error. The standard error of an effect can be estimated from replicate measurements at nominal levels, duplicated experiments in the design, higher order interaction effects, etc.

TABLE 1 Design matrix for a 2^3 factorial design, in which the influence of the factors pH, temperature, and percentage organic modifier is studied on the resolution as the chromatographic response

	Factors			
Run	pH	%ACN	Temperature	Resolution: yi
1	−	−	−	Y1
2	+	−	−	Y2
3		+	−	Y3
4	−	+	−	Y4
5	−	−	+	Y5
6	+	−	+	Y6
7	+	+	+	Y7
8	−	+	+	Y8

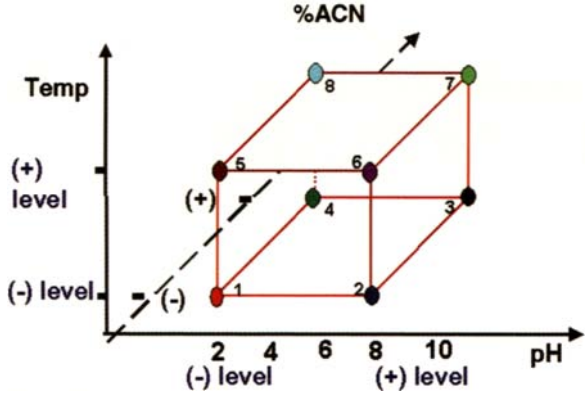

FIGURE 4 The geometric representation of the 2^3 full factorial design, corresponding with Table 1.

In Figure 5, the minimum resolution plot (the smallest resolution value that is obtained for all possible peak pairs in the mixture of studied compounds) is shown for the separation of a mixture of chlorophenols obtained after the application of a Doehlert design.[14] In this design, the separation of the chlorophenols was studied as a function of the pH and the percentage of acetonitrile in the mobile phase with only seven experimental runs. The pH ranged from 3 to 7, whereas the percentage of acetonitrile ranged from 42% to 56%. As can be observed, a robust optimum is predicted around pH 5.5 and 49% acetonitrile. The predicted optimal conditions were verified and resulted in the chromatogram with an acceptable separation as is shown in Figure 6.

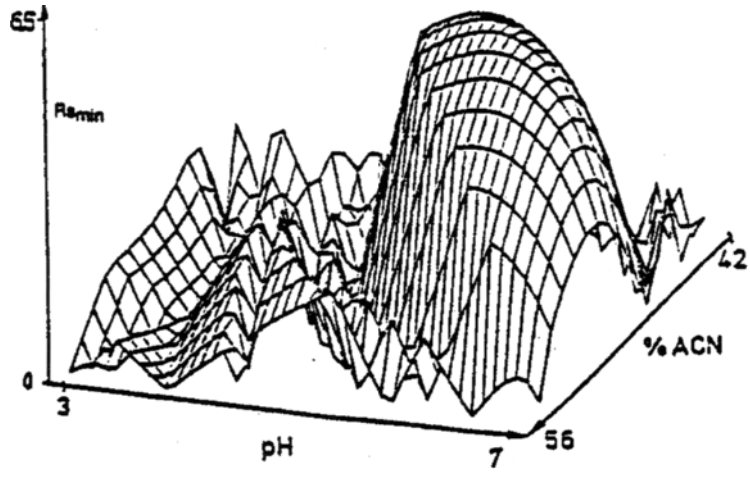

FIGURE 5 Three-dimensional graph of the minimum resolution of all possible peak pairs for a mixture of chlorophenols, as a function of pH and concentration of acetonitrile. (Reprinted from reference 14 with permission.)

3. Computer-Assisted Approach

A good understanding of the chromatographic separation allows for effective method development. Many rules that relate chromatographic elution to changes in the separation conditions have been described.[8,19–21] For example, the relationship between retention and the percentage of organic modifier in reversed phase HPLC can be applied to make accurate predictions of separation conditions.[18,19,22] In the case, where a theoretical modeling is not feasible, empirical modeling is applied based on a limited number of practical experiments.[14] There are many software tools available that assist the analyst during the development of an HPLC method.[8,23] Usually, the most important factors to be optimized for an HPLC method are the characteristics of the stationary phase, the mobile phase pH and composition, the gradient profile, and the column temperature. The column stationary phase and the mobile phase pH are selected based on the characteristics of the solutes, but the other factors generally require thorough optimization. Therefore, software programs that allow optimization of more than one chromatographic variable simultaneously are very useful. Moreover, by applying method optimization software tools, additional advantages can be obtained: the technology is traceable from analyst to analyst or from lab to lab (files retrieve/sent); there is a reduction of "trial and error" experiments that allows saving time and costs; and a better understanding of the separation process through evaluations of simulations. As a result, robust methods (transfers) are obtained. Examples of frequently applied software tools are DRYLAB (LC Resources,

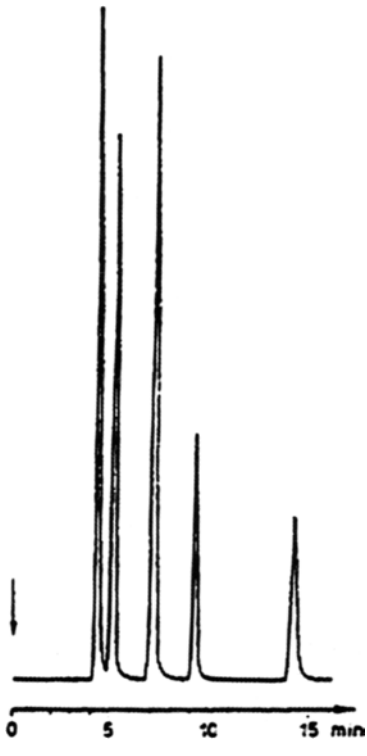

FIGURE 6 **Practically obtained chromatogram for a mixture of chlorophenols at the predicted optimal conditions of 49% acetonitrile and pH = 5.5. (Reprinted from reference 14 with permission.)**

Walnut Creek, CA), CHROMSWORD (Merck, Darmstadt, Germany), ACD-LC simulator/Chromatography laboratory (ACDLABS, Toronto, Canada).

Figure 7a (with the compliments of Dr. Koen Vanhoutte, Johnson and Johnson Pharmaceutical Research and Development) shows a chromatogram with the separation of a main compound: MC and its four impurities by a gradient elution method on a C-18 BDS column using ammonium acetate-acetonitrile in the mobile phase. As can be observed, impurity 1 elutes on the tail of the main compound and requires the method to be optimized for a more robust separation. The optimization was performed by the aid of DRYLAB, in which the gradient profile and the column temperature were optimized, simultaneously. The generated minimum resolution plot for this application as functions of the gradient time and the column temperature is shown in Figure 7b. As can be observed, acceptable separations are predicted at conditions with a gradient time >15 min. The temperature effect is also very important since at lower gradient times, higher temperatures are required for better

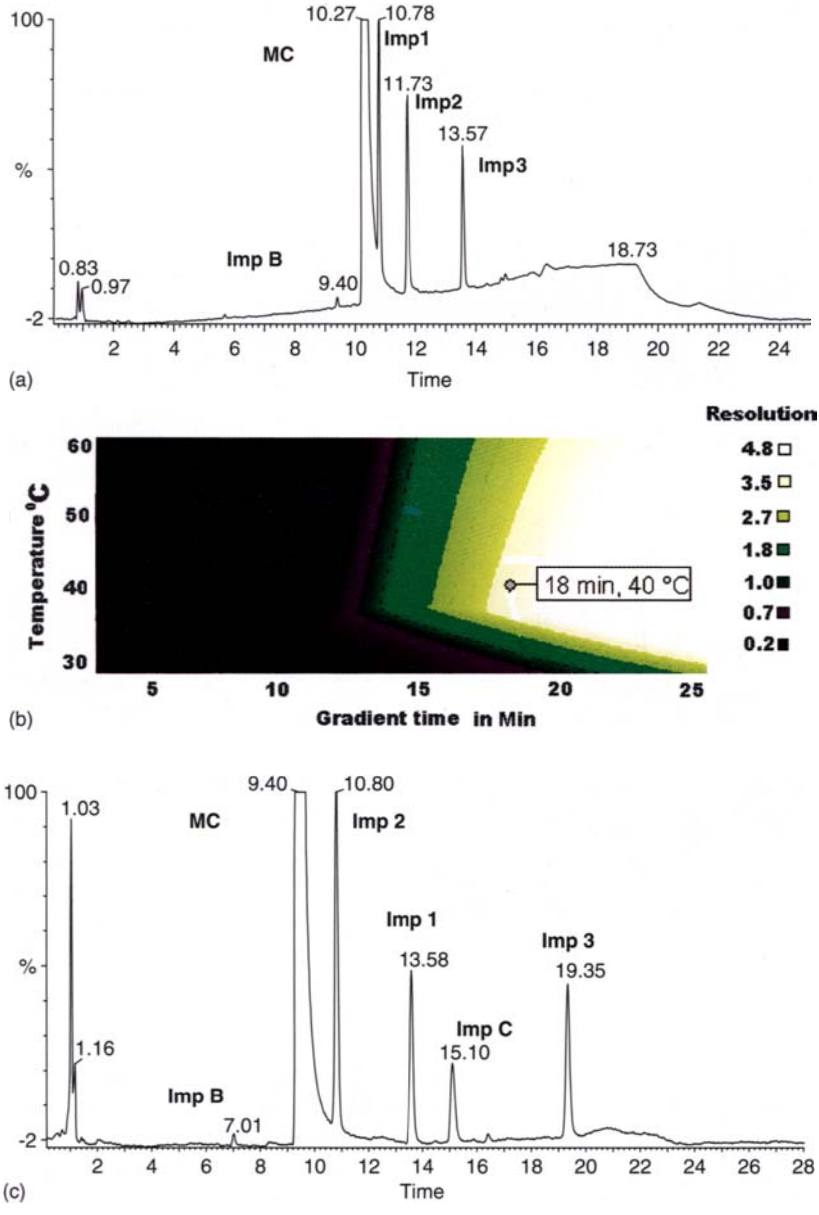

FIGURE 7 (a) Early development HPLC gradient elution method for MC and four related impurities. The separation between MC and Imp 1 required further optimization. (b) Drylab minimum resolution plot for the simultaneous optimization of the separation as functions of the temperature and the gradient time. As can be observed, optimal conditions are predicted at gradient times of more than 15 min and at higher temperatures. At lower temperature, longer gradient times are required. Optimal conditions were selected at 18 min and 40°C. (c) Practically obtained chromatogram at the predicted optimal conditions of 18 min gradient time and 40°C. As can be observed, all the compounds are nicely separated. Additionally, a new impurity was identified.

TABLE 2 Prediction Accuracy Between the Calculated Chromatogram and the Practically Obtained HPLC Chromatogram for a Linear Gradient Method

Peak	DryLab prediction (RRT)	Experiment (RRT)
Imp B	7.70 min (0.77)	7.00 min (0.74)
MC	9.99 min (1.00)	9.40 min (1.00)
Imp 2	11.28 min (1.13)	10.80 min (1.15)
Imp 1	13.88 min (1.39)	13.58 min (1.44)
Imp C	15.04 min (1.51)	15.10 min (1.61)
Imp 3	19.35 min (1.94)	19.35 min (2.06)

resolution. Optimal separation conditions were selected at a gradient time of 18 min and a column temperature of 40°C. The experimentally obtained chromatogram at these conditions is presented in Figure 7c. As can be observed, the impurity in the peak tail is nicely separated from the main compound, resulting in a very good separation of MC from impurity 1.

Additionally, a new unknown impurity is detected that co-eluted with the main compound peak in the previous method. It is important to note that peak tracking is crucial when applying computer-assisted method development. The correct assignment of peak identity will determine the accuracy of the predicted separation. A way to achieve this is by applying MS-detection during method development. As can be seen from Table 2, the DRYLAB-predicted retention times corresponded very well to the experimental values. The optimal separation conditions should be optimized further for robustness and transferability.

B. Stress Studies

When the late phase method is to be applied for the evaluation of product stability, the method has to be stability indicating. For this reason, stress samples have to be generated and analyzed with the method. This evaluates the selectivity of the method toward potential degradation products. Stress samples are generated by degradation of the active pharmaceutical ingredient and traditional DP formulations, typically according to conditions as depicted in Table 3. Placebo mixtures are also run as part of the study to identify any excipient degradation pathways. Changes or revisions to existing analytical methods that are considered major and that require extensive validation are subject to stress studies to confirm that the method is stability indicating.[24,25]

TABLE 3 Conditions for Stress Testing

Product	Stress parameters	Storage conditions[a]
Drug substance in solution	Acidic conditions	Room and elevated temperature
	Basic conditions	Room and elevated temperature
	Oxidative conditions	Room and elevated temperature
	Reductive conditions	Room and elevated temperature
	ICH light conditions	
Drug product in solid state	40°C/75% RH	
	50–60°C/ambient RH	Open dish
	ICH light	

[a]Degradation is a function of storage time. 10–15% degradation is targeted.

All potential selectivity challenges in the HPLC chromatograms of the stressed samples should be evaluated. Typical co-elution of specified impurities are easily detected by an orthogonal specific detection technique like LC-MS and often solved by proper chromatographic column and conditions selection.[26] The presence of highly retained potential degradation compounds (e.g. dimers) are also investigated, although this is not as easy. Indeed, verification that all compounds being injected are also eluting from the column is demanding, since the chromatography is the limiting factor. This selectivity challenge can be solved by combining the aqueous conditions with non-aqueous reversed phase conditions in one continuous chromatographic run. Other critical regions in the chromatogram are the void volume and potential co-elution with the API. Whereas the non-retained compounds (eluting with the void-volume) show a significantly increased polarity, potential co-eluters with the API are generally constitutional and stereoisomers of the active ingredient. Both of these two issues may be evaluated using an orthogonal capillary electrophoresis method.[26]

C. System Suitability Tests and Limits

In each method, system suitability tests are incorporated to ensure consistent performance in day-to-day operation. A system suitability test (SST) is an integral part of many analytical methods[27] and ascertains the suitability and effectiveness of the operating system.[28] The SST limits for the different parameters are usually established based on the experimental results obtained during the optimization of a method and on the experience of the analyst. However, the ICH guidelines recommend that, "one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g. resolution tests) are established to ensure that the validity of the analytical procedure is maintained whenever used." It is necessary to include a test to examine the column and system performance.

TABLE 4 Parameters Generally Applied in a System Suitability Test (SST) With Typical Limits for Drug Substance (DS) and Drug Product (DP) Methods

Parameter	Limit DS	Limit DP	Comments
System repeatability	0.73% RSD	2.0% RSD	For at least five replicate injections of a reference solution(s)
Reporting threshold	*X*% (w/w) to be defined on an ad hoc basis	*X*% (w/w) to be defined on an ad hoc basis	Main compound peak should be readily integrated (recovery between 50% and 150% compared to the 100% API
Accuracy	98.5–101.5% recovery API	98.0–102.0% recovery API	Tested by analysis of a second reference solution[a]
System drift	98.5–101.5% recovery API	98.0–102.0% recovery API	Tested by injecting a control reference solution after injection of the samples[a]

[a]Other tests may be used.

1. Column Performance

The selectivity is demonstrated by analysis of a mixture of the relevant compounds, e.g. a selectivity batch/sample solution. The resulting chromatogram is compared with the selectivity chromatogram provided in the method description. The resolution between the critical peak pair(s) is determined. The target resolution is 2.0 (the calculation expression is recommended to be mentioned in the method description). In cases where there is no critical peak pair, the tailing factor of the API is used as an SST parameter instead of resolution. The limit for the tailing factor is dependent on the application and is preferentially set based on the results of a robustness test.

2. System Performance

System precision and reporting threshold checks are applied as system performance tests. The parameters and recommended limits for DS and DP for late phase methods are compiled in Table 4.

D. Method Performance Evaluation

As can be inferred from Figure 2, before extensive validation the performance of the method is evaluated appropriately. Column durability tests, robustness testing for the chromatographic and sample preparation conditions, analytical method evaluation ring tests (AMERTs), method capability assessments, and pre-validation studies are applied to challenge the late phase method. During method evaluation studies the QC labs play an important role, since they will have to apply the method for a long time in the future. Method evaluation tests will be discussed later in this chapter.

The late phase method is validated according to the applicable regulatory guidelines.[27,29,30] As depicted in Figure 2, method validation is not considered to be the most important activity in the method development process (only ±15% of the development time). The validation is performed according to a standard operating procedure and at this stage is expected to be completed successfully. Any method shortcomings should have been detected during the actual development process. After validation, the method is transferred to the receiving laboratory. The development laboratory will provide proper support and training to the receiving laboratory in order to facilitate a smooth transfer. The transfer process is described in a separate standard operating procedure and is expected to be completed seamlessly, since the receiving laboratories are already familiar with the late phase method because of their continuous involvement. The QC labs will monitor the performance of the method during the lifetime of the method. Several method performance indicators are recorded each time the method is applied. These indicators can be related with column performance, System suitability test results, number of OOS results and deviations related to the method. The feedback on method performance is crucial for the development lab and will be used to drive subsequent method development processes.

1. Column Durability Studies

Once the chromatographic method is developed, the selectivity of the optimized separation is evaluated on at least three different lots of the selected stationary phase for the selected column. The goal is to check whether the selected stationary phase (globally available) is suitable to be applied in a final method.

The results are qualitatively evaluated by making overlay plots. An example for an HPLC gradient separation for a typical pharmaceutical product in which the main compound and its related substances are analyzed is shown in Figure 8a. As can be observed, apart from two minor selectivity issues (indicated by the arrows in Figure 8a), the reproducibility on different lots of stationary phases is quite acceptable. Here the selectivity is evaluated on five different lots of the selected stationary phase. Besides the study of different lots of the stationary phase, a durability test is performed on one column by making at least 100 consecutive injection runs of a reference and a sample (preferably an old sample) solution.

The result is qualitatively evaluated by making overlays of the sample solutions. Figure 8b shows such an example in which more than 300 injections were performed continuously in a single sequence. In the example, chromatogram the main compound and its related compounds are separated using a gradient elution method using a Waters Xterra™ MS C_{18} column (3.5 µm particle size, 4.6 × 100 mm); with a pH 9.0 mobile phase (ammonium acetate–diethylamine) as solvent A and a mixture of

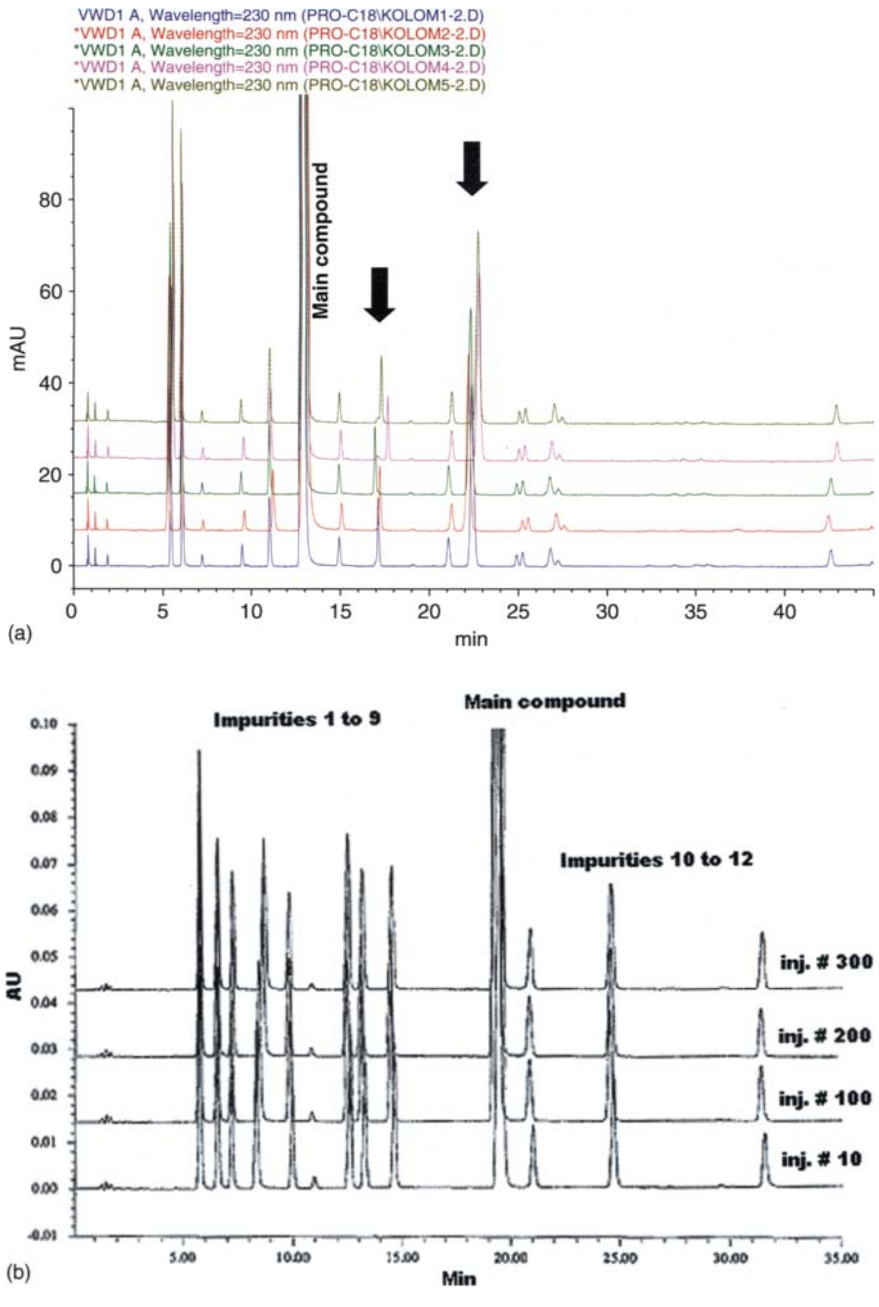

FIGURE 8 **(a) Overlay chromatograms of a typical gradient elution separation of a main compound and its related impurities on five HPLC columns with different lots of the stationary phase. There are only slight shifts (arrows) in retention times detected. (b) Overlay of the 10th, 100th, 200th, and 300th chromatograms of a typical gradient elution separation of a main compound and its related impurities on a single HPLC column obtained after consecutive injections in a single sequence. There is almost no shift in retention times detected for the eluting compounds.**

acetonitrile and 2-propanol as solvent B. The flow rate was set at 1 ml/min., the column temperature at 35°C, injection volume at 10 µl and UV detection at 275 nm. As can be observed in the overlay chromatograms there is hardly any peak shift to be detected, indicating stable run conditions for the chromatographic method.

2. Robustness Studies

The robustness of the chromatographic and the sample preparation conditions should be evaluated. A detailed discussion is provided in Chapter 16.

E. Pre-Validation Evaluation Studies

After method optimization the most important aspects of the validation are checked in a pre-validation study to examine whether the method is ready for evaluation at the customer site. If necessary, method optimization may need to be repeated. Suggested parameters to be checked may include: specificity/selectivity against placebo, stressed placebo, stressed samples, relevant impurities and degradation products, verification of the reporting threshold, linearity check of the API, filtration studies, stability of solutions, method comparison by evaluation of batch results obtained with the final and early development methods, and a small precision study by analyzing a batch in triplicate. It is recommended to check peak purity of the API by using LC-PDA and LC-MS.

V. METHOD EVALUATION PHASE

The analytical method development process is continued with additional method evaluation tests conducted with direct involvement of the customer (receiving laboratory). The goal is to check whether the late phase method performs adequately in different labs.

A. Analytical Method Evaluation Ring Test

The AMERT is a critical component of the analytical method development process as it allows for verification of whether a method performs adequately for the intended purposes and complies with the specific country requirements with respect to analytical methods. The concept of performing the AMERT was introduced by Crowther et al.[1] and has been applied extensively within J&J Pharmaceutical Research and Development. The AMERT enables the development laboratory to take into account the valuable feedback from the receiving laboratories on the method performance and is therefore scheduled prior to the time-consuming validation (see also Method Development Process – overview

in Section V). An additional goal is to facilitate a seamless transfer of the methods immediately following the validation. AMERT is carried out for analytical methods (usually chromatographic procedures) that are intended to be subjected to parallel testing during transfers. In some cases, AMERTs may be necessary for starting materials, key intermediates, and critical raw materials. Post approval, the receiving laboratory may have to upgrade filed analytical methods. The AMERT is part of the method development process and the outcome can affect the final method description. For this reason, the AMERT must be performed after the method robustness testing and prior to the extended method validation preceding method transfer. Due to the preset target dates for filing, the provided time frame for the completion of the AMERT is frequently rather restricted. A target time period of 3 weeks should be considered to cover all activities related to the AMERT. This implies a close collaboration between the involved laboratories.

B. Method Capability Assessment

MSA[2] aims to assess the performance of the analysis method in its application environment. It is explicitly chosen to refer to a system, because the outcome of an analysis is determined not only by the quality of the method description, but also the equipment, reagents, the lab environment, analysts, understanding of procedures, definitions, nomenclature, etc. MSA is performed by applying a Gage R&R study that investigates the precision and the discriminative ability of the measurement method and relates this with process variation and the product specifications. The Gage R&R study will determine if the analytical method can discriminate differences in the measured properties (e.g. assay value) of the batches (parts) and therefore can be used for process improvement/control and for acceptance testing. The repeatability and reproducibility of the measurement system is typically estimated from a designed trial across different batches and labs. Since products are tested according to analytical method descriptions, the complete analytical method (i.e. from sample receipt to result) has to be evaluated. In the Gage R&R study, a randomized block design is applied, in which the two major factors (analyst and batch) are switched and randomized. As is shown in Figure 9, the analytical variance that is observed in analytical results obtained for the analysis of different production batches, is the total variance due to the method and to the production process. In order to determine the contribution of the method variance (apart from that of the production process variance) an ANOVA method is applied.

Additionally, by applying a Gage R&R study it is possible to assess the reproducibility and the method capability for process improvement/control

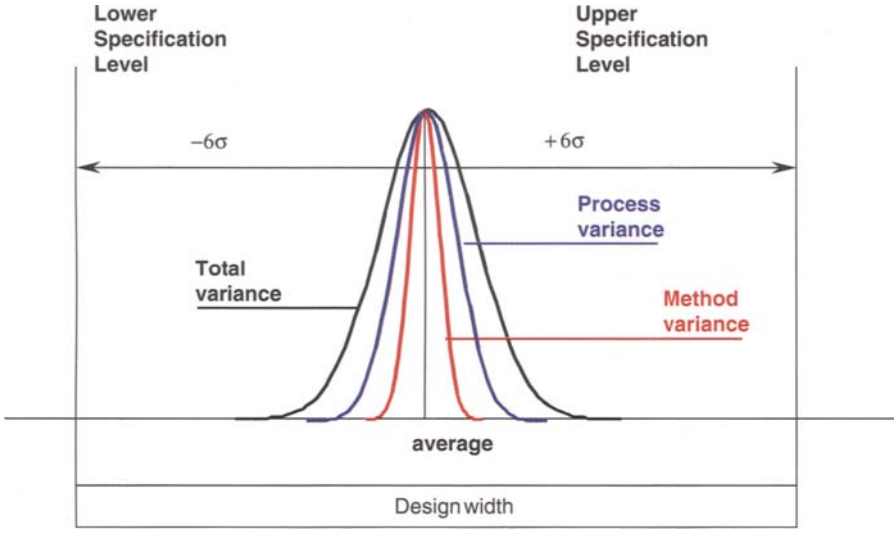

FIGURE 9 **Diagram indicating that the total variance in the analysis results equals the sum of the method variance and the process variance. A capable measurement system has a method variance that is less than 30% of the design width (difference between upper specification level and lower specification level). The production process is considered to be under full control when the average assay value is centered at 6 sigma values away from the lower and upper specification levels.**

and for acceptance testing against specifications. The following metrics are calculated:

- The %R&R value (repeatability and reproducibility). This addresses the portion of the observed total process variation that is taken up by the measurement error. The %R&R value is an important metric for the method capability toward application in process improvement studies.

$$\%\text{R\&R} = \left(\frac{s_{\text{MS}}}{s_{\text{Total}}} \right) \times 100$$

where s_{MS} is the overall standard deviation of analytical method, and s_{Total} is the observed total process variation (method component included).

- The %P/T value (precision-to-tolerance ratio). This addresses what portion of the specification is taken up by the measurement error. The %P/T value is an important metric for the method capability toward application in release testing against specifications.

$$\%\text{P/T} = \frac{5.15 \times s_{\text{MS}} \times 100}{(\text{USL} - \text{LSL})}$$

where USL is the upper specification limit and LSL is the lower specification limit.

1. Study Set Up

The samples (minimum five batches) included in the study should cover the expected normal variation of the process (target ± 3 sigma). If the batches used do not represent the full and normal process variation, the calculations are based on a historical value for process variation. The same batches are analyzed by multiple analysts (minimum two) in different laboratories (minimum three) using their own instruments, reagents, and solvents. Each analyst performs the entire method as described. Every sample should be analyzed at least twice (with independent sample preparation) in the same run. The replicates should also be blinded and randomly tested.

As an example, the results of a Gage R&R study performed for a oral film coated tablet in four different labs, where six sample batches were analyzed in replicate by two analysts in each lab are presented in Table 5. The %P/T metric was calculated according to the Ph. Eur[30] (specifications: 95–105%) and the USP[31] (specifications: 90–110%). The method standard deviation (excluding the process standard deviation) is also presented, together with average assay results and confidence intervals thereof per lab and for all labs together.

The %R&R of 52.9% implies that approximately half of the observed total process variation is taken up by the measurement error. This percentage is mostly caused by the repeatability (difference between the replicates by the same analyst). The %P/T of 50.4% implies that when testing against the specification limit of 95.0–105.0%, half of the specification width is taken up by the method imprecision. This means that there will be a high risk of unnecessarily rejecting batches when analyzed with this method against the considered specifications. The method is more capable of being applied for testing according to USP specifications (90.0–110.0%), since a %P/T of 25.2% is obtained. For lab 1, which

TABLE 5 Results of a Gage R&R Study for a Typical Oral Film-Coated Tablet Assay Procedure

Laboratory	%R&R	%P/T(Ph. Eur) for 95–105%	%P/T(USP) for 90–110%	STDEV Method	Mean assay	95% CI
Lab 1	32.6	28.3	14.1	0.55	97.2	±1.10
Lab 2	49.6	55.0	27.5	1.07	96.6	±2.14
Lab 3	55.1	48.8	24.4	0.95	96.3	±1.90
Lab 4	56.1	46.0	23.0	0.89	96.1	±1.78
Total	52.9	50.4	25.2	0.98	96.5	±1.96

STDEV, standard deviation; CI, confidence interval.

was the development lab, a better method capability is obtained compared to the application labs 2, 3, and 4. Lab 1 has developed the method and therefore knows the method in full detail. Usually, the development lab works under the most optimal conditions for the method, resulting in better performance. The difference between the two types of labs may be an opportunity for the application labs. By proper training, guidance, and support both types of labs should be able to achieve comparable performance.

The overall method standard deviation is quite good (0.98%) and is mostly caused by analyst variation. Such a level of precision is typical for HPLC DP assay methods and will be difficult to improve further. The required effort and costs of reducing the method variance further will be high and technically very demanding to the QC labs (high payoff in an effort for a little extra quality). One way of reducing the analytical variation further is by increasing the number of sample preparations. For example, to detect differences of 2% between batches using a standard deviation of the analytical method of 1.0% and a product variation of 0.7% (resulting in a total standard deviation of 1.2), a sample size of 6 should be used. For differences of 3%, four samples are sufficient and for 4%, three samples should be used. Moreover, when the average assay value of the produced batches is at 100%, the impact may be minor, but when there is an offset in the content of main compound in the produced batches there is a higher probability that batches will be rejected.

2. Gage Run Chart

An example of a Gage run chart is shown in Figure 10 in which all measurement raw data are depicted in one graph. This is an example for a Gage R&R study of an HPLC method for the determination of active in an oral film-coated tablet formulation. The method was intended to be transferred to four different QC labs, in different global locations. The study is performed on six samples with two replicates by eight analysts (two different analysts in four different laboratories). From this visualization, the reproducibility and repeatability of the method is immediately observed. For example, it can be observed that all lab average results are well correlated. The repeatability of the method appears to be less uniform (e.g. results of analyst 2 in lab 2).

VI. METHOD TRANSFER PHASE

After validation the method is formally transferred to the application labs for use during the entire product life cycle. In the classical approach, the method transfer study was considered to be the real challenge of the method. Moreover, it was at this stage that the customers were involved in the process. As a result, many issues were encountered at the transfer

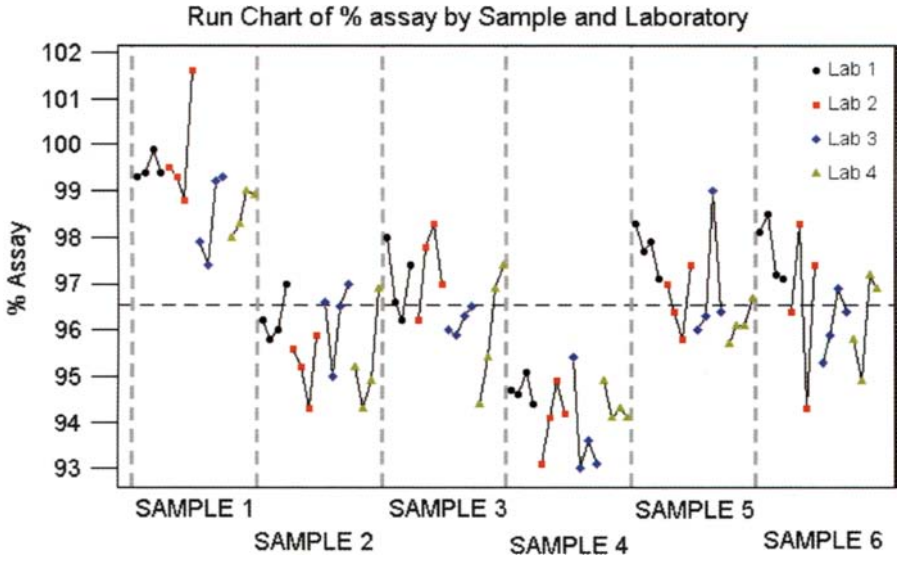

FIGURE 10 **Gage run chart of an HPLC method for the determination of active in an oral tablet formulation. Six samples are analyzed with two replicates by two analysts per lab at four different sites. For each lab, four results are plotted; the first two are from analyst 1 and last two results are from analyst 2.**

and were required to be resolved. With the continuous involvement of the customers, the final method is not "new" for the application labs. They have participated in the requirement setting of the method and in the evaluation of the method. Using this approach, the application labs have already tried the method out in their own environment and issues on method applicability have been resolved prior to the transfer study.

Method transfer is considered to be a formal process to demonstrate equal method performance between the development and the application labs. After a successful transfer, it is established that the application lab is qualified to apply the final method for release of products. For a smooth execution, it is recommended to prepare a document package containing all relevant information for the transfer with regard to product safety, product specifications, analysis methods, validation reports, method development background, analytical technical requirements, and the transfer protocol. The transfer protocol will describe product identity, overview of test methods, acceptance criteria, responsible personnel, and specific rationales (e.g. selection of tests for parallel testing).

For each test, it is advised to analyze the samples with replicates (e.g. $n = 6$). The acceptance criteria are based on the method purpose and on the validation characteristics. A recommended approach would be to propose acceptance limits based on the results of the Gage R&R study.

If not performed for any particular reasons, one can refer to the Horwitz equation[11] that relates method repeatability with method reproducibility. Typically, the difference in average assay values for DS methods should be within 2.0% and the precision should be less than 1.0% RSD in each lab. For DP, these limits are 3.0% and 2.0% for average assay difference and for the precision in each lab, respectively. The impurities are usually considered at a 0.5% level and the typically allowed difference between labs should be less than 0.2% (absolute) with a within-lab precision of less than 15% RSD in each lab.

A transfer report is written to complete the transfer process. The report typically consists of product information, reference to a completed protocol and validated method, a summary of the transfer results with references to both the raw data, and a statement on the successfulness of the transfer. Any deviations to the protocol must be recorded. Unsuccessful test results should be discussed, documented, and investigated. The action plan and follow-up results should be properly documented. The transfer is closed with the sign-off of the report and, depending on the success of the transfer, a qualification statement for the application lab.

VII. METHOD PERFORMANCE MONITORING AND FEEDBACK

Monitoring the performance of methods during real-time analysis will generate a historical source of objective data and will be the basis for future discussions on the method between the application and development labs. For the development lab, the objective data are a valuable source to detect method shortcomings and to find out which aspects of the method should be improved. Moreover, the method feedback will be used to steer the method development process in general. The feedback on method performance is generally discussed regularly (at least once a year). During the monitoring, a number of key performance indicators are recorded and filled out in feedback sheets by the application labs (stability and operational labs) each time the method is applied. The method feedback sheet is sent together with the method description to the application labs at transfer. A completed example of such a feedback sheet is shown in Figure 11.

Several method performance indicators are tracked, monitored, and recorded. Items that are recorded include: the date of analysis, identification of the HPLC system, identification of the analyst, number and type of samples analyzed, the system precision, the critical resolution or tailing factor, the recovery at the reporting threshold level, the recovery of a second reference weighing, the recovery for control references (repeated reference injections for evaluation of system drift), the separation quality, blank issues, out of specification issues, carry over issues,

Laboratory: Stability Evaluation and Release
Method: R115777 F032 - F034 - Drug product
Date: Start July 2001

Key Process Output Variable Feedback Sheet

General					SST								OOS	Deviations SST failure			
Date	System type	Initials Analyst	number of samples	Sample type	RSD	R	T	RT-check	Ref 2	Control references	Selectivity (separation pass/fail?)	Blank peaks (Y/N)	OOS reference number	NC reference number	Carry over Y/N	Other causes	Comments
23-Jul-01	AO-0442	KMAE	4	F32_F34	0.20	6.6	1.6	98	99.8	99.8	PASS	Y			N		KB101113
06-Aug-01	AO-0435	DDEK	6	F32_F34	0.10	4.2	1.6	89	99.4	99.5	PASS	N			N		KB101118
07-Aug-01	AO-0435	DDEK	18	F32_F34	0.10	4.3	1.6	95	99.6	99.9	PASS	N			N		KB101119
21-Aug-01	AO-0445	DDEK	6	F32_F34	0.10	6.2	1.9	80	100.0	99.8	PASS	N			N		KB101126
04-Sep-01	AO-0444	ADES	12	F32_F34	0.10	7.0	1.7	114	100.9	99.8	PASS	Y			N		KB101138
12-Sep-01	AO-0445	DDEK	12	F32_F34	0.10	6.4	1.5	101	99.8	99.8	PASS	Y			N		KB101140
18-Sep-01	AO-0445	DDEK	4	F32_F34	0.30	6.3	1.6	98	99.8	100.0	PASS	Y			N		KB101144
18-Sep-01	AO-0442	DDEK	6	F32_F34	0.20	6.7	1.6	93	99.7	100.2	PASS	Y			N		KB101145
19-Sep-01	AO-0445	DDEK	6	F32	0.10	6.2	1.6	104	99.8	100.1	PASS	Y			N		KB101147
20-Sep-01	AO-0445	DDEK	18	F32	0.20	6.1	1.6	98	99.6	99.8	PASS	Y			N		KB101149
20-Sep-01	AO-0442	DDEK	6	F34	0.10	6.6	1.5	93	99.8	100.0	PASS	Y			N		KB101150
21-Sep-01	AO-0442	DDEK	18	F34	0.20	6.7	1.5	99	99.8	100.2	PASS	Y			N		KB101152
27-Sep-01	AO-0445	DDEK	16	F32-F34	0.20	6.2	1.6	104	100.0	99.9	PASS	Y	LO111059		N		KB101156
02-Oct-01	AO-0445	DDEK	4	F32-F34	0.20	6.5	1.6	102	99.7	99.8	PASS	Y			N		KB101159
03-Oct-01	AO-0445	DDEK	12	F32-F34	0.20	6.4	1.6	106	99.9	99.8	PASS	Y			N		KB101160
12-Oct-01	AO-0445	DDEK	8	F32-F34	0.20	6.4	1.6	98	99.9	100.2	PASS	Y			N		KB101167
15-Oct-01	AO-0445	DDEK	6	F32-F34	0.50	6.5	1.6	103	99.7	100.0	PASS	Y			N		KB101168
16-Oct-01	AO-0445	DDEK	18	F32-F34	0.20	6.4	1.5	100	99.5	100.2	PASS	Y			N		KB101169
26-Oct-01	AO-0445	DDEK	2	F32	0.10	6.3	1.5	101	99.8	99.9	PASS	Y		NC112440	Y		KB101178
14-Nov-01	AO-0445	DDEK	10	F32-F34	0.10	6.3	1.6	103	99.6	99.7	PASS	Y			N		KB101206
28-Nov-01	AO-0445	DDEK	18	F32-F34	0.40	6.4	1.6	100	99.8	100.4	PASS	Y			N		KB101224
03-Dec-01	AO-0445	DDEK	18	F32-F34	0.30	6.4	1.6	98	100.0	99.8	PASS	Y			N		KB101229
05-Dec-01	AO-0444	DDEK	8	F32-F34	0.10	6.6	1.7	105	99.6	99.8	PASS	N			N		KB101231
		Upper Limit			2.00		2.6	150.0	102.0	102.0							
		Lower limit				3.0		50.0	98.0	98.0							

FIGURE 11 Example of a completed feedback sheet.

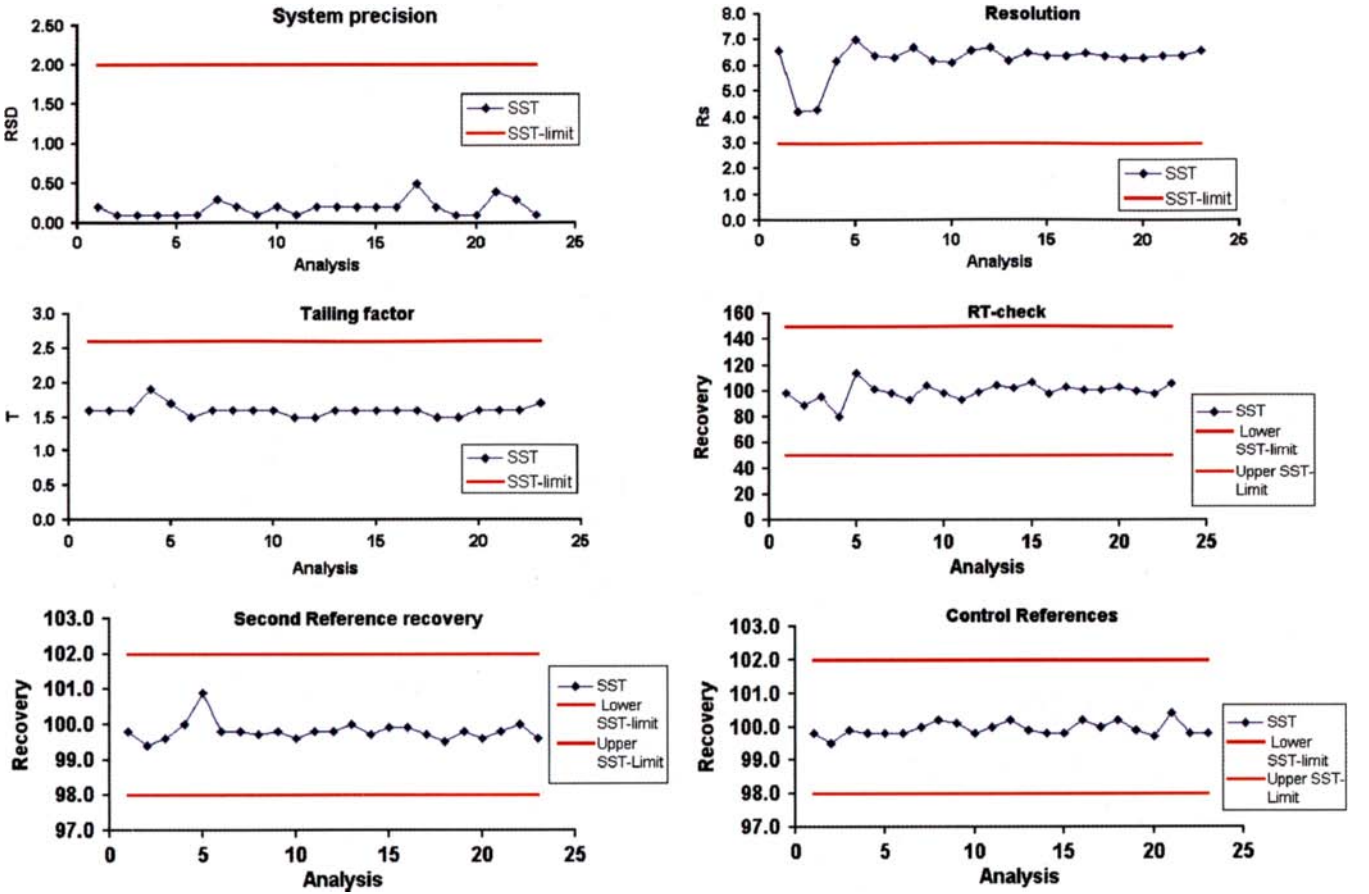

FIGURE 12 Control charts of six quantitative method performance indicators.

and other non-conformances. The quantitative indicators are additionally visualized by plotting control charts (Figure 12). As can be observed in Figure 12 the system precision for half year time period (23 analyses sequences) is always below 0.5% RSD. Additionally, the second reference, the control reference and the reporting threshold level have quite stable recovery values.

VIII. METHOD DEVELOPMENT CYCLE TIMES AND DOCUMENTATION

In order to reduce cycle-time it is encouraged where possible to perform the different steps of the method development process in a parallel sequence instead of a serial approach. This can be achieved by including more analysts in the method development activities. The following documents are suggested to support the developed method:

- Informal method development plan
- Method definition requirement document
- Method development summary
- Detailed test method description
- AMERT protocol and report
- Gage R&R protocol and report
- Method validation protocol and report
- Method transfer protocol and report
- Completed method feedback form

IX. CONCLUSIONS

Method development is a process in which both the development lab and the receiving lab have to work closely in order to generate a test method that is well documented, easily understood, and simply performed by an appropriately trained analyst. Within QC environment it is not important to have the scientifically most fancy method; a technically straightforward, easy-to-use and most robust, and GMP-compliant method is preferred. After a successful development, validation, and transfer, the method development lab should still be kept accountable for the performance of the method. Tracking of the output is key to understand where gaps are located. Additionally, discussions on method shortcomings are then based on facts and figures, allowing for a better partnership between the development and the receiving labs. Understanding gaps results in learning of the method development process that in turn is favorable for improvement in future method development endeavors.

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14

USE OF HPLC FOR IN-PROCESS TESTING

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ABSTRACT

HPLC is widely used for in-process testing in both drug substance development and production as a control for ensuring that processing steps have gone to completion. A variety of examples are used throughout this chapter to highlight the different areas where in-process testing is used and how the information obtained influences a particular synthetic pathway. Recent advances in Fast HPLC allow information to be obtained within minutes which can significantly speed up the development process.

I. INTRODUCTION

Various types of in-process tests or controls (IPCs) are used throughout the pharmaceutical industry to ensure that intermediate steps are complete prior to the end of a process. The main goal of conducting an IPC is

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to ensure that at the end of the process, material of acceptable quality and yield will be produced. During the manufacture of drug substances (either in research, development or in manufacturing) this can involve a number of analytical techniques depending on which stage of the process is being monitored. A typical chemical process may consist of any of the following steps: reaction, work-up, separation, crystallization, and isolation/drying. Each step may have an in-process test to ensure that it has been completed to a level which will allow the process to continue successfully. Alternatively, the IPC will raise an alert that the process has not been completed successfully and that further action is required prior to proceeding to the next phase. In cases where IPCs do not meet the specifications, corrective actions differ depending on the environment the test is being carried out in. In production the regulations are more strict, and a material which does not meet the specifications has to be either re-processed by repeating a prior step that is part of the process or re-worked by processing the material through a known filed re-work procedure. Also, it is always easier if such a material can be re-processed by repeating a normal production step. In addition, if a process repeatedly fails an IPC the process itself may need to be altered (and re-validated) such that the IPC will be met.

The choice of whether an IPC is needed is made based on the experience gained in the development and subsequent manufacturing using the process. For each process as part of the quality documents, a list of IPCs and their expected results are available. Depending on the type of IPC the action taken in the event of a failing IPC will also vary. For drying steps, samples are often taken for loss on drying (LOD) to ensure that drying is complete. Typically, if a batch is not dried to an acceptable level the drying process will continue until passing results are obtained. The same is true for reaction completion IPCs. Sometimes a process will require a longer reaction time than expected and in the event of a failing IPC the batch would just be allowed to react longer. Other processes may require additional reagents to be added. Still other reactions may add reagents in portions taking IPCs after each portion to avoid over-reaction or addition of an excess of reagent which could be difficult to remove. When failing IPCs are encountered, which is not expected, one should always consider the sample and the analysis as these are also areas where errors can occur. In some production plants, sampling devices which use vacuum to extract the sample into a sampling port are used in lieu of opening the manway to the reactor and oftentimes problems are encountered with these devices especially if they are not properly washed from the last sample. Sometimes just re-sampling the reaction mixture will lead to the expected result. Similarly, there are always cases where problems are encountered in QC and out of specification (OOS) results are obtained through analytical or instrument error. When a failing IPC is obtained a proper investigation into the legitimacy of the result should be carried out prior to an investigation in the plant. For manufacturing processes the acceptable actions that can be taken (without

compromising quality) will be outlined in a special quality document as part of the handover from development to production.

Once a process has been transferred to production, the details of how the process is run including any IPCs will all be part of the dossier which is filed with the appropriate health authorities. However, changes can be filed with the health authorities and this can happen for different reasons throughout the lifetime of the product. For processes which have been running for long periods of time there are cases where enough data can be collected to justify eliminating IPCs in order to speed up process times. For example, if a given process has an IPC for reaction completion and it can be shown that the IPC has never failed then that IPC can be phased out or only taken at the discretion of the responsible chemist. Eliminating unnecessary IPCs can save hours, depending on how the IPC is carried out.

In contrast to chemical development and production, IPCs in pharmaceutical production are very different due to the nature of the processes involved. In pharmaceutical processes batches of drug substances are combined with different excipients to produce dosage forms such as tablets and capsules. A typical pharmaceutical process may include blending, granulation, compression or encapsulation, and coating steps. These steps in the process may have IPCs but they are different from those carried out in chemical processes.

II. VARIOUS EXAMPLES OF IN-PROCESS TESTING IN DRUG SUBSTANCE DEVELOPMENT

Development activities for a particular compound will typically begin when enough evidence has been obtained in research to warrant larger quantities of material for further studies. In research, the focus is to produce relatively small amounts of compound quickly for further research activities. The amount of in-process testing here will be minimal and may consist of either a TLC or an HPLC analysis to ensure the reaction is complete.

In contrast, development activities focus on producing larger quantities of drug substance for pharmaceutical and clinical development. During the different stages of development different levels of quality are required with regulations becoming stricter during the later phases. In manufacturing where material is made exclusively for human use, chemical processes must be validated and this includes in-process testing. As IPCs are more common in development and manufacturing, our discussions will focus on these areas. In early phase development, there are four main situations where IPCs are used: evaluating reaction conditions, process monitoring for reaction conversion, evaluating purification steps (crystallization and/or chromatography), and determining purity of the final compound. In later stages of development other tests such as impurity profiles, particle size, and LOD and/or residual solvents during drying phases may become

important. Almost all types of in-process testing for chemical reactions involves some chromatographic technique.

In early stages of development, procedures for individual reactions will be provided from research for the synthetic route for a particular compound. Sometimes, small samples that are used as references may be provided. At the research stage, the purity of a compound is not well defined. Development work will start with an evaluation of the research route to the compound. Each step of a synthesis will be carried out first on a relatively small scale (1–2 g or smaller). These small-scale experiments are used to determine if the reaction itself is suitable for larger scale production, if the reaction conversion is sufficient, and if the purity and yield are both acceptable. If necessary, changes to the original synthesis will be made for any number of reasons. If reference samples are provided these will be used to develop HPLC procedures for purity. During this evaluation phase, IPCs will consist of using these HPLC procedures to ensure that a particular reaction is complete and estimate the purity of the desired compound prior to working up the reaction. This will also influence whether or not a purification step is required. If the purity is low, the compound may be worked up and then purified by either column chromatography or crystallization. Once necessary purification steps are complete, the HPLC method can be used again to determine the final purity. It should be noted that typically at this stage, purity is calculated by area percent and is not purity relative to a standard. These initial analytical results will form the basis for comparison when additional batches are produced.

A. Evaluating Reaction Conditions

In early stages of chemical development some reactions do not proceed with satisfactory yield and purity. In these cases it is necessary to do some scouting experiments to find the best conditions to optimize these parameters. An HPLC method will be developed for starting material and product, and various sets of conditions will be evaluated to determine those which give the best reaction conversion. It is important to note that sometimes adequate time is not always available to develop "perfect" conditions. Two examples which illustrate this point will be described.

The first example is of a reduction reaction where the desired product has two stereocenters. The reduction reaction would give a diastereomeric mixture. The starting material and product are shown in Figure 1a and b. The starting material and product were well separated by HPLC, and different reaction conditions and reagents were chosen to determine the best set of conditions to optimize the conversion. The HPLC of the product also shows an additional peak of about 5%, by area, that could not be completely removed. The starting material is an alkylated compound and the alkylation proceeds from either the top or the bottom face of the molecule and a 1:1 mixture is obtained. The subsequent reduction results in four diastereomers. In this particular case we wanted to maximize the selectivity

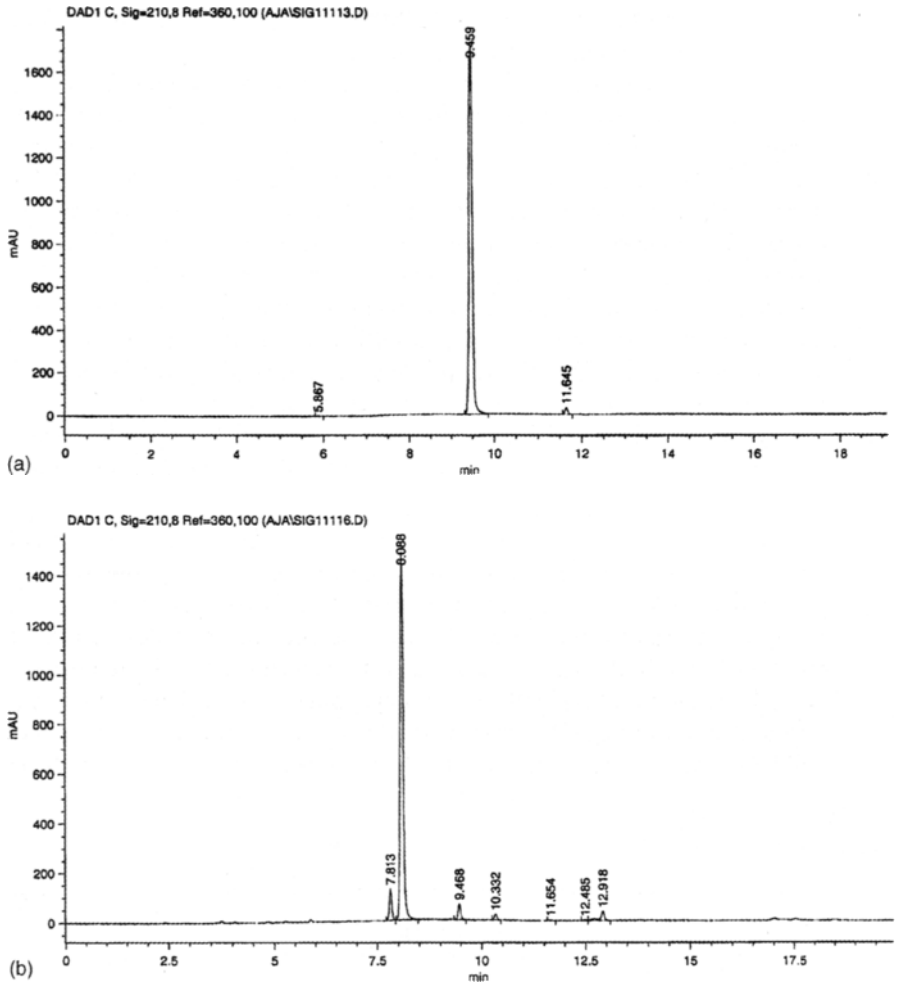


FIGURE 1 **(a) Starting material for the transformation. (b) Product of the reduction reaction. (c) Reaction conversion using LAH as the reducing agent. (d) Reaction conversion using sodium borohydride as the reducing agent. (e) Reaction conversion using DIBAL at 0°C. (f) Reaction conversions using DIBAL at −75°C.**

so that one diastereomer was obtained. In order to do this three different reducing agents were screened: lithium aluminum hydride (LAH), sodium borohydride ($NABH_4$), and diisobutyl aluminum hydride (DIBAL). For these initial experiments the solvent chosen was tetrahydrofuran and no further solvent screening was carried out. Figure 1c shows the result of using LAH as the reducing agent. A ratio of approximately 53:40 was obtained with more of the undesired diastereomer. Switching the reducing agent to $NABH_4$ gave similar results, 54:44, again with the wrong diastereomer in greater amounts (Figure 1d). The best results were obtained with DIBAL. At 0°C we obtained a ratio of 16:61. However at this temperature

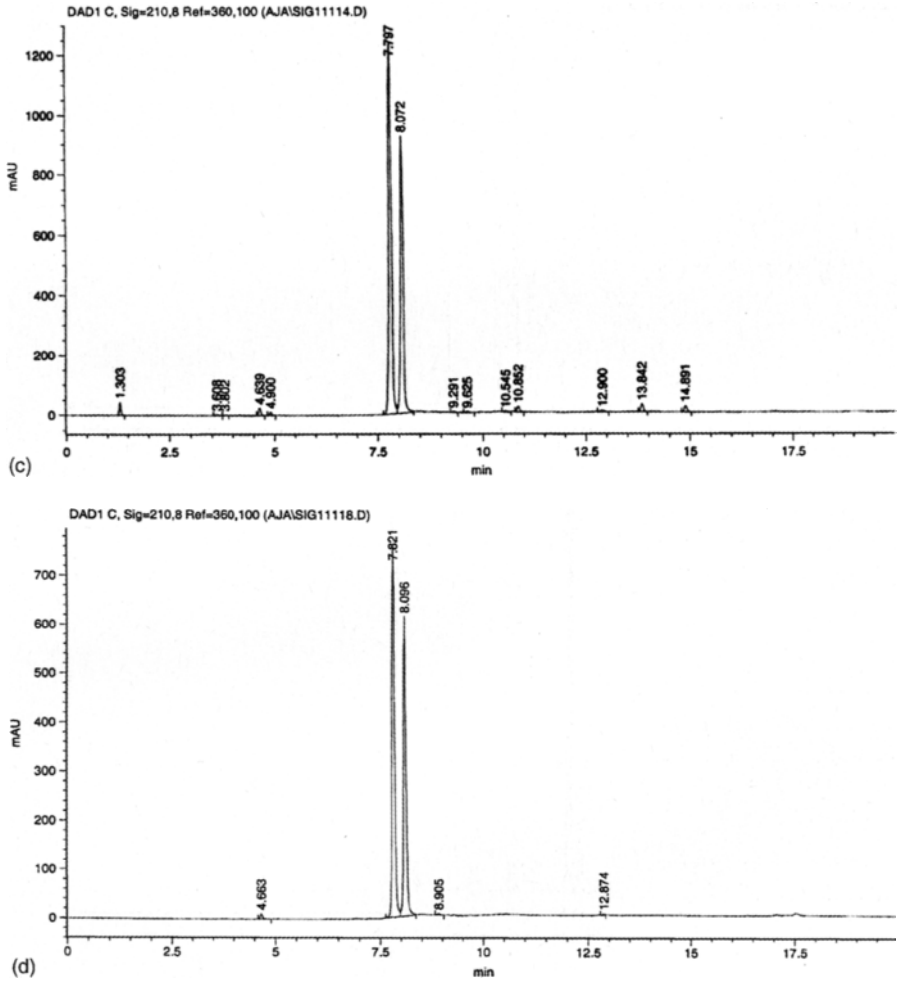


FIGURE 1 (Cont.)

there was also approximately 20% starting material remaining and the reaction did not go to completion (Figure 1e). Decreasing the temperature to −75°C gave us the best conditions for this transformation and a ratio of 11:87 with no starting material present (Figure 1f).

B. Examples of Process Monitoring for Reaction Conversion

Once the best conditions are chosen for a particular reaction, the reaction can then be scaled up using these conditions. A good example of a reaction that was monitored is one similar to the general reaction scheme shown in Scheme 1.

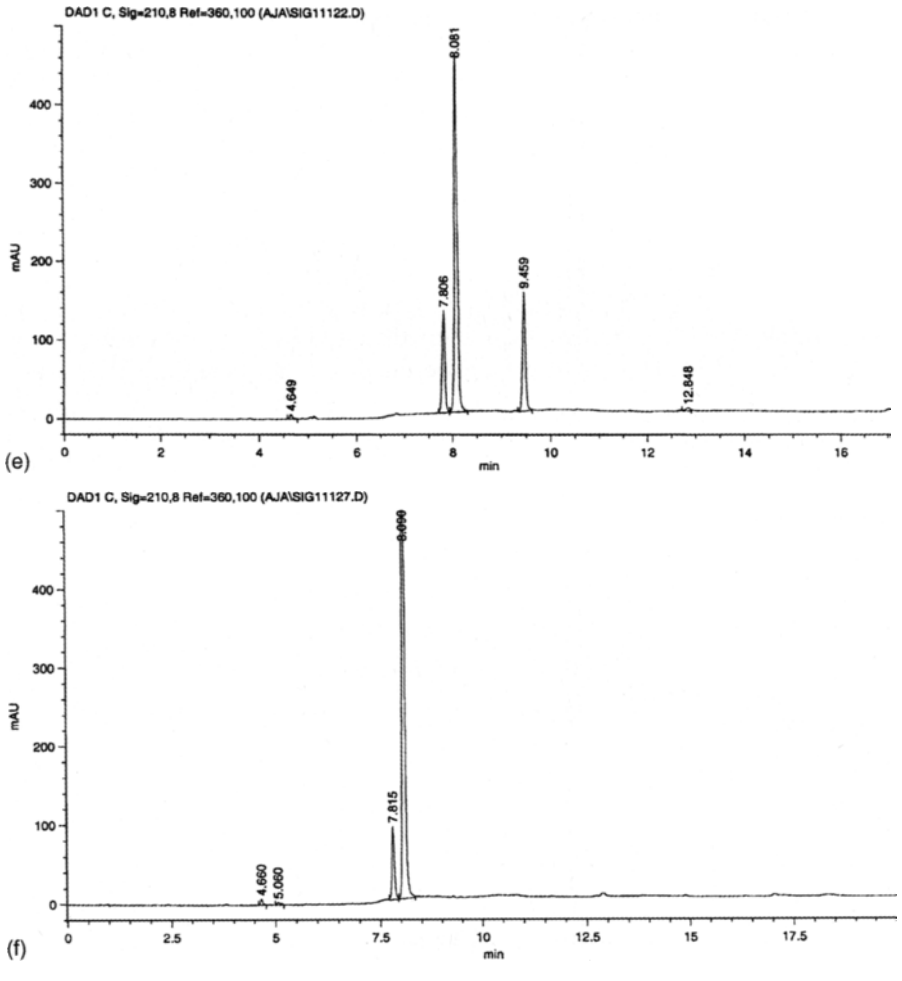
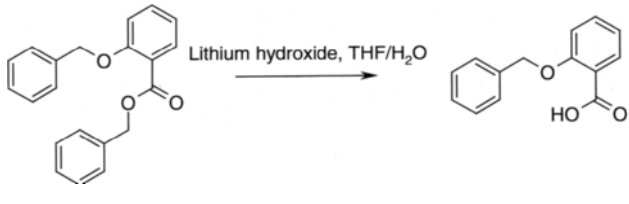


FIGURE 1 (Cont.)



SCHEME 1 General scheme for removal of a benzyl protecting group.

A laboratory scale experiment was carried out on 250 g of material. Reference samples had been provided for both starting material and product and an appropriate HPLC method was developed. Figure 2 (top) shows the chromatogram corresponding to the starting material and Figure 2 (bottom) shows the chromatogram of the product. Both compounds are well separated with the system chosen and this method was used to monitor the reaction. This particular reaction was carried out overnight and the following morning a sample was taken for an IPC. Figure 3a shows the chromatogram in which approximately 6% of starting material is still present. Monitoring was continued in this way with samples for IPC taken at 1–2 h intervals until less than 1% starting material remained. This small-scale experiment set the stage for a later larger

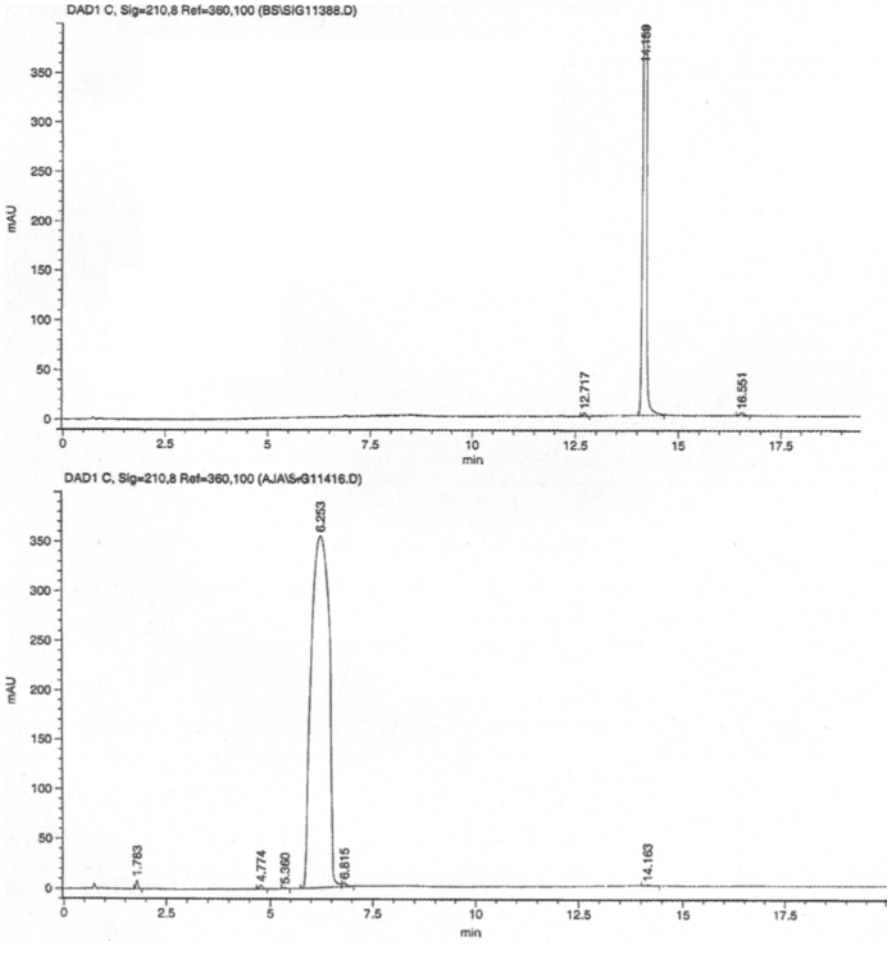


FIGURE 2 **Top: Chromatogram of starting material. Peak at 14.1 min. Bottom: Chromatogram of product. Peak at 6.253 min.**

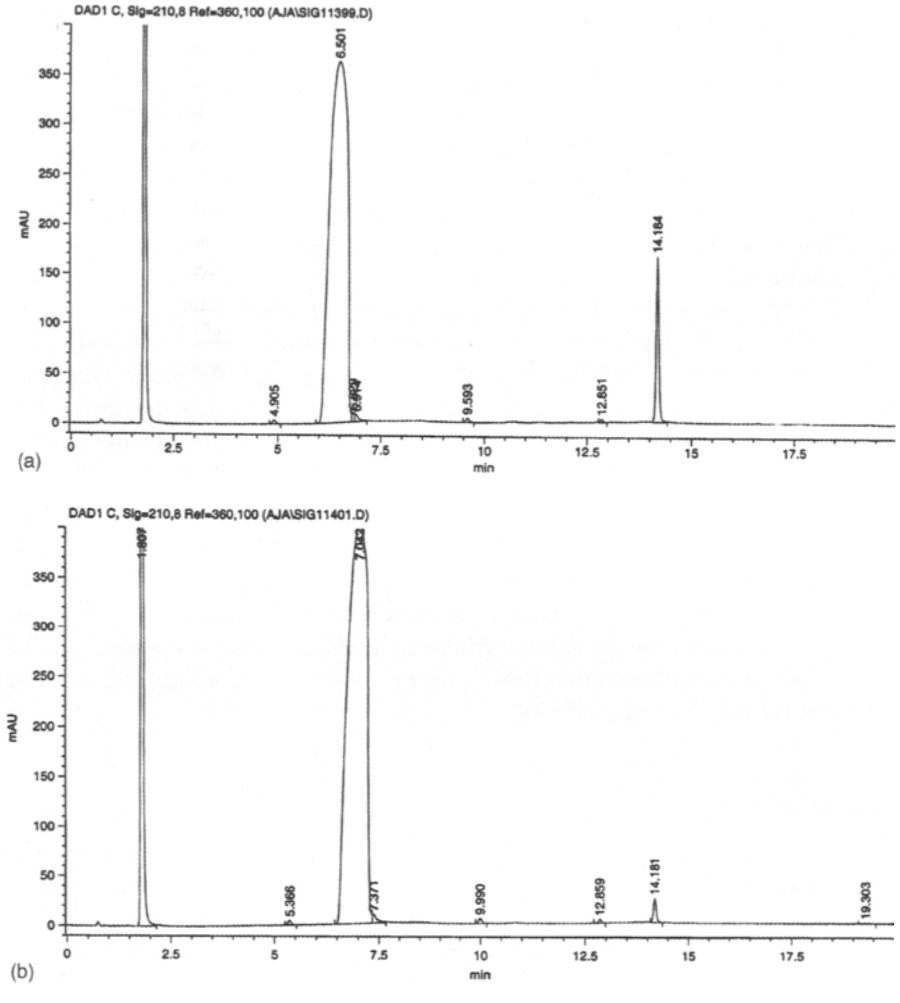


FIGURE 3 **(a) IPC chromatogram showing 92% product and 6.6% starting material remaining. Peak prior to 2.5 min is toluene. (b) IPC chromatogram at reaction completion; less than 1% starting material present. Peak at 1.8 min is most likely benzyl alcohol.**

scale batch which was produced. We now knew approximately how long the reaction would take to reach less than 1% starting material and took our sample for IPC at the appropriate time. The purity of our compound at the end of the reaction was approximately 97% and following a crystallization procedure we were able to increase the purity to 99% (Figure 3b).

Initially, method development will consist of finding the best chromatographic technique where a separation between starting material and product can be achieved. HPLC is the most commonly used method but in cases where it is unsuccessful, GC, TLC and in rare cases NMR are also used.

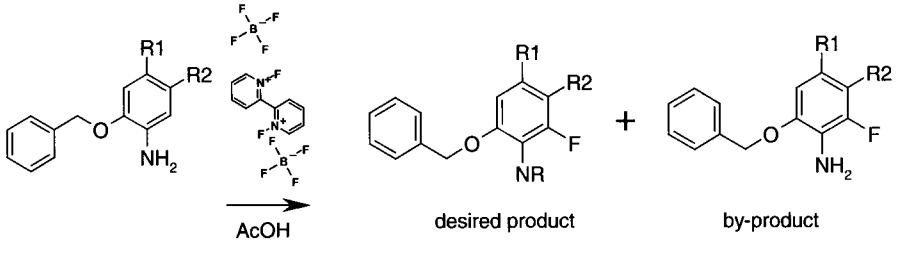
FIGURE 4 General structure of a lactone where R2 is aromatic.

Another interesting example of reaction monitoring came from a hydrogenation reaction on a lactone whose general structure is shown in Figure 4.

Hydrogenation of lactones of this type to the open form is well known in the literature and a variety of catalysts have been evaluated. During the reaction three bonds are hydrogenated to obtain the open form and depending on the conditions the reaction can go through a number of intermediates. To complicate matters, our specific compound also had a protecting group that was sensitive to hydrogenation and was also hydrogenated. During the evaluation phase a number of different catalysts were evaluated with each one progressing through a different route. By the end of the process development when all the information was evaluated we were able to analyze how the reaction progressed and had identified all of the intermediate products visible by HPLC. This allowed us to determine how long the reaction had to run in order to obtain the desired product.

C. Evaluation of Purification Steps

As previously mentioned, different methods are used for in-process testing depending on where the testing is being carried out and the level of sensitivity required for the results. There are other situations where alternate methods are advantageous and times where two methods can be used in conjunction with each other. In this example, TLC was used preliminarily as a tool to select appropriate fractions from column chromatography for HPLC evaluation. The transformation carried out was similar to that shown in Scheme 2.



SCHEME 2 General structure of the fluorination reaction to the desired product and the by-product.

The reaction conversion proceeds such that the desired compound could not be separated from a reaction impurity by crystallization. A column chromatographic method was developed first using TLC which was then scaled up to a plant-sized column of 80 kg of silica gel in order to obtain pure product. Chromatograms of the starting material (Figure 5a) and the reaction mixture are shown below (Figure 5b). During the chromatography, TLC was used to monitor the fractions. Those which had similar TLC profiles were collected together, evaporated, and then submitted for HPLC analysis for purity. This reduced the number of samples requiring analysis by HPLC to only those for which high purities were for the most part guaranteed. In the end the product was isolated

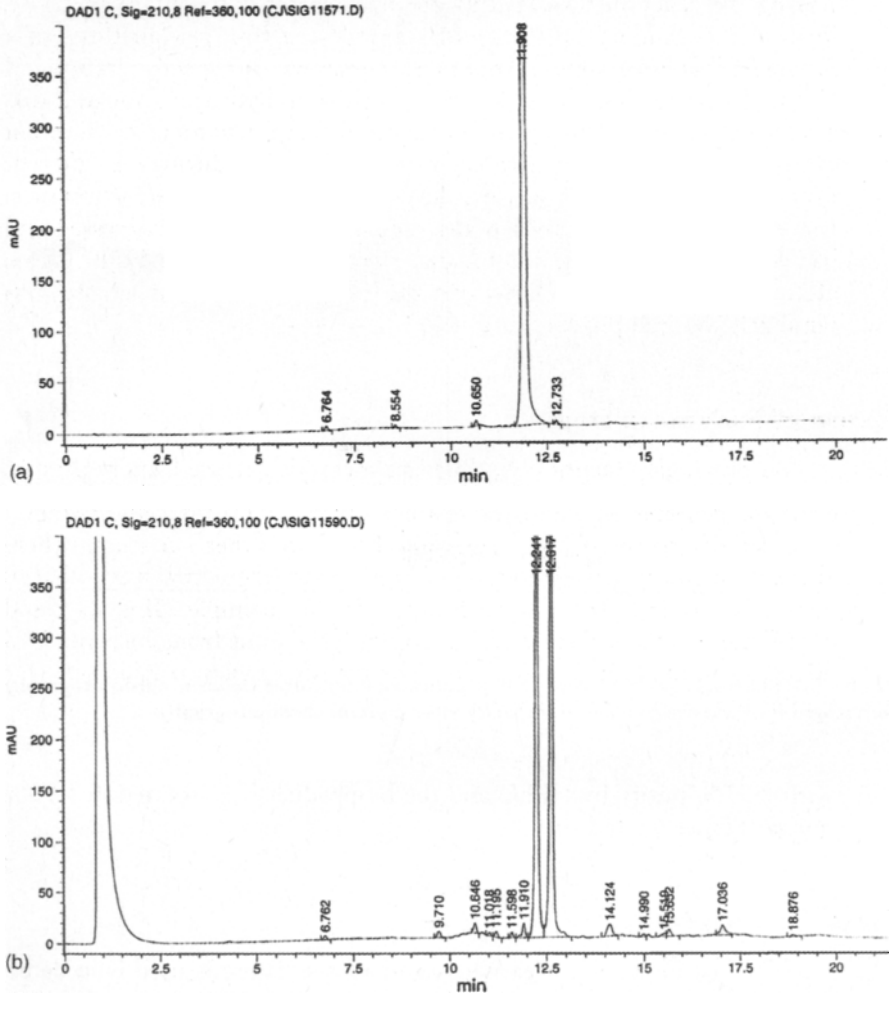


FIGURE 5 **(a) Chromatogram of the starting material. (b) Chromatogram of the reaction mixture.**

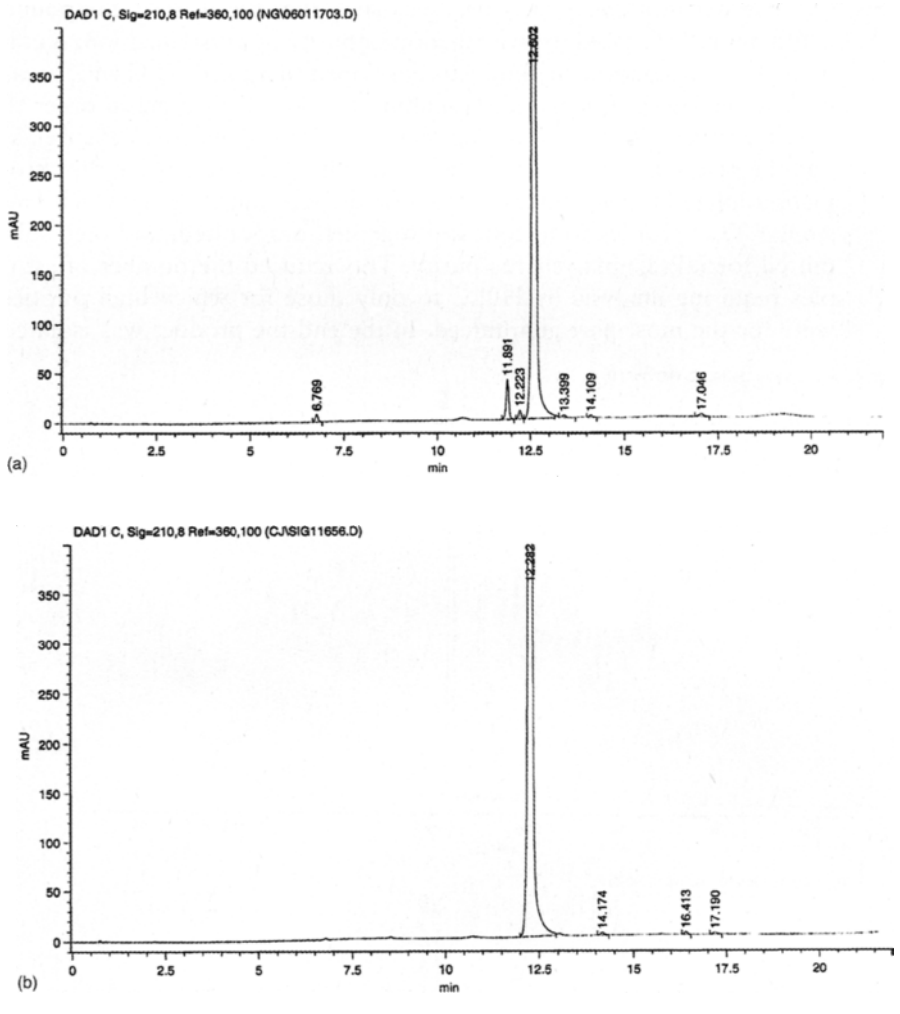


FIGURE 6 **(a) Chromatogram of the pure product isolated after column chromatography. (b) Chromatogram of the main reaction impurity after column chromatography.**

with >97% purity by HPLC and the by-product was isolated in >99% purity (Figure 6a and b).

D. Determining Final Purity

The last important area where in-process testing is used is in determining the final purity of a particular material. This is done when a crude reaction mixture is used in the next step or a purification process takes place. In this example the crude reaction mixture had a purity of

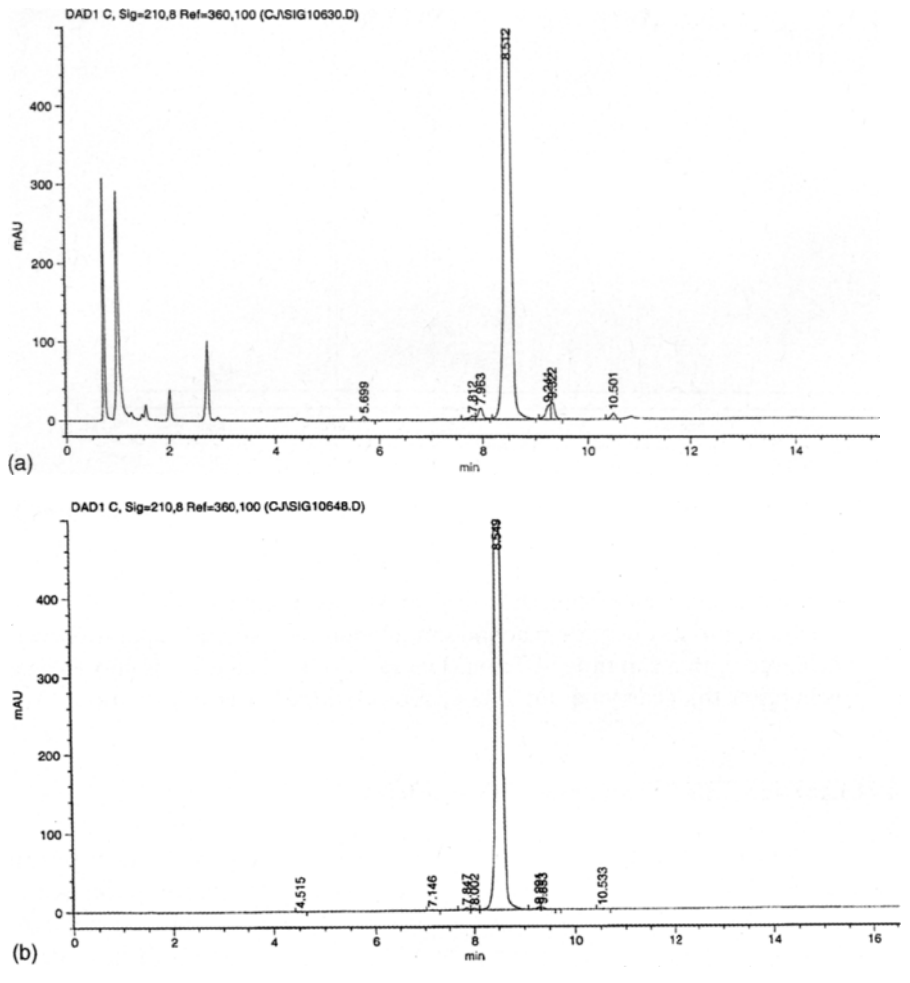


FIGURE 7 **(a) Crude reaction mixture. (b) Pure compound after crystallization.**

about 95% (Figure 7a) and through crystallization we were able to increase the purity to 99% (Figure 7b). Sometimes crystallizations, chromatographies, or extractions are used to remove inorganic impurities that may not be detected by HPLC. In the example above, the reaction used a palladium catalyst and the crystallization was used not only to improve the purity but also to reduce the amount of palladium in the product.

III. FAST HPLC

An alternative to conventional HPLC is "Fast" HPLC.[1] Fast HPLC uses shorter columns with monolithic packing materials to provide separations

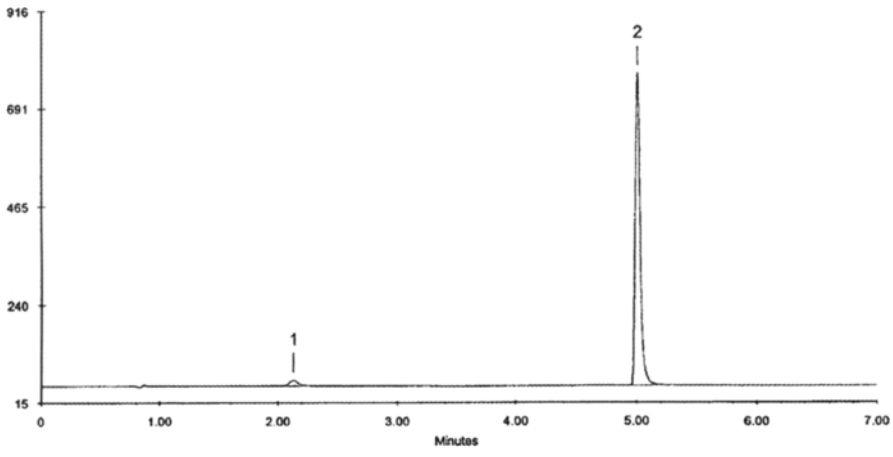


FIGURE 8 **Fast HPLC chromatogram showing starting material (peak 1) and product (peak 2) with a 7-min run time.**

in much shorter times than those previously observed. Fast HPLC was used for an in-process test for reaction completion and a good separation was achieved with a run time of 7 min (Figure 8). This method was chosen as it was faster than carrying out a TLC and provided better information.

IV. IN-PROCESS TESTING IN CHEMICAL PRODUCTION

Once a process has been transferred from development to chemical production it is ready to be produced on a kilogram to ton scale. Depending on the demand for the product the process may be run continuously in high volumes or it may only be run periodically in campaigns. For high-volume products a lot of experience can be gained in a short period of time especially if multiple batches are produced weekly.

Regardless of whether a process is run continuously or in campaigns with careful and appropriate monitoring, data can be collected to support the need for or the elimination of an in-process test The goal of production is to reduce as much as possible any unnecessary time that can be eliminated from the production process (without compromising quality or yield).

For example consider the following sample process where data was collected from in-process tests for reaction completion (Figure 9). The IPC test is carried out by HPLC and monitors the amount of unreacted starting material remaining at the end of the reaction. A campaign of 15 batches was produced and for every batch of the campaign the level of unreacted starting material was well below the acceptable limit of 2%.

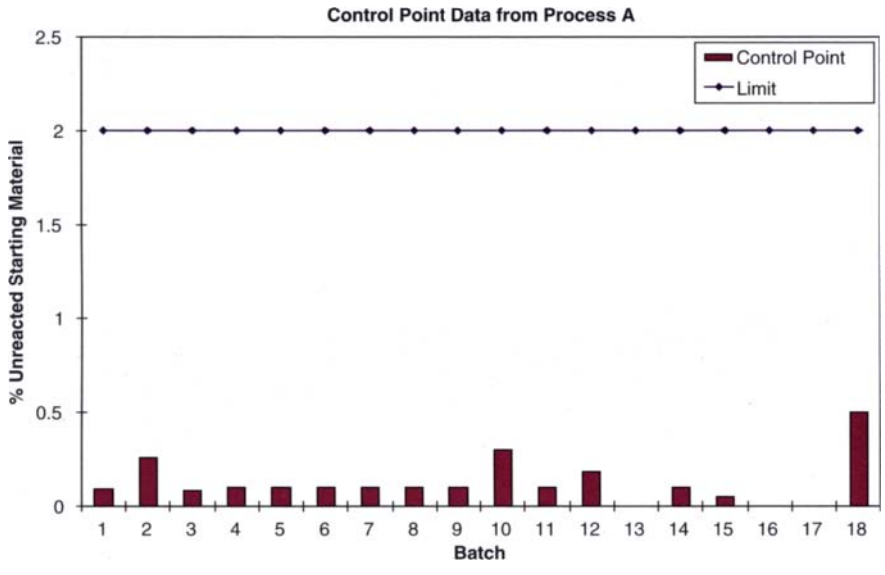


FIGURE 9 **Graph showing sample data from Process A for reaction completion. Data for each batch is well below the limit of 2%, making this process a good candidate for eliminating the IPC.**

This process would be a good candidate for eliminating the IPC or at the very least testing for reaction completion only when there has been a deviation from the normal process that would likely affect the reaction completion.

A typical IPC carried out by HPLC takes anywhere from 0 to 2 h depending on how long it takes to sample the vessel and deliver the sample to the lab, for the testing to be carried out, and for the results to be calculated and in some cases reviewed. If there are problems with testing, the 2-h period can easily become longer and the process will have to wait until the results are available. In addition, some reaction mixtures are difficult to sample and there are situations where the sampling alone can take time and cause problems.

There are other situations where IPCs are put into a process as a result of a processing problem that occurred in the plant. When this happens, the details of the origin of the problem may not be known at the time and an IPC may be necessary to allow production to continue until the problem is fully understood. Once all the facts have come to light, the IPC may no longer be necessary. Consider the second example for Process B where an IPC was taken for monitoring of an impurity which occurred as a result of the undercharge of a particular reagent. This IPC was put in "for information only" and an investigation into the details of how the impurity formed was carried out. Enough data was collected to support only sampling for this particular IPC if an undercharge of this

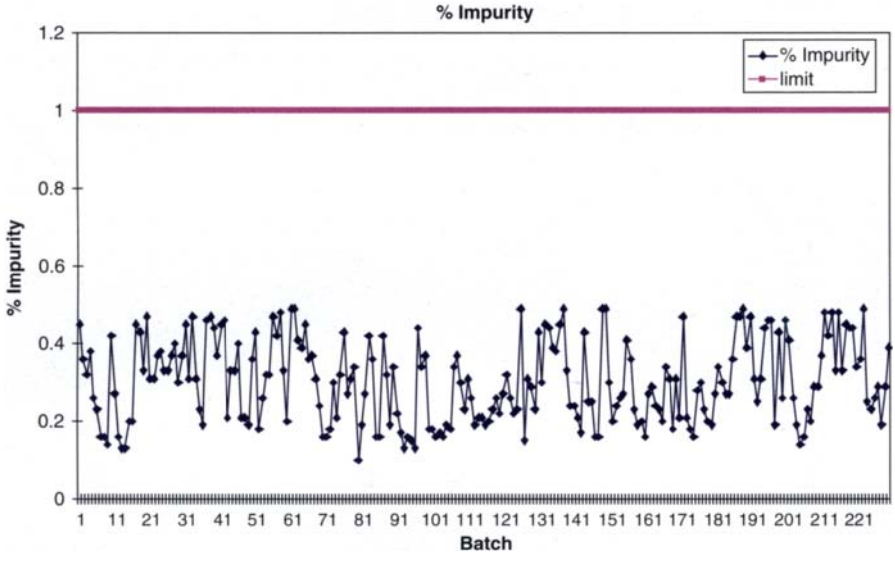


FIGURE 10 **Data for impurity monitoring for Process B. Sample data for over 200 batches demonstrating the process was well in control and the IPC was no longer required under normal processing conditions.**

particular reagent was suspected (Figure 10). This significantly shortened the reaction times for the process and lightened the workload of the IPC laboratory. In this situation, in lieu of eliminating the test outright, we chose to remove it in stages where we tested every other batch for a period of batches. As we gained confidence in the process we progressively reduced testing until it was only carried out at the discretion of the responsible chemist.

V. IN-PROCESS TESTING IN PHARMACEUTICAL PRODUCTION

Pharmaceutical production also uses in-process testing but as the processes are dramatically different, the testing required for normal batch production is also different. Typical pharmaceutical processes may involve blending, granulation, compression or encapsulation, and coating steps. During validation of the different process steps, samples will be pulled at different time points for content uniformity, assay, and dissolution testing and this is normally carried out by HPLC. During normal production, in-process testing will be limited and is typically carried out on the production floor. Hardness of the tablets may be checked at various intervals to ensure that the tabletting machine is working properly and in some cases, disintegration will be carried out.

VI. CONCLUSIONS AND FUTURE DIRECTIONS

In-process testing is a very important aspect of pharmaceutical development and production. As timelines in both areas come more and more under scrutiny, the time allowed for this testing is becoming shorter and shorter. Fast HPLC is likely to be employed in more areas in the future to help develop processes quickly and to shorten production times. Another area of interest which is becoming more popular is Process Analytical Technology (PAT).[2] PAT is a way of carrying out on-line monitoring of processes and completely eliminates sampling and testing. The most common uses for PAT are in drying and crystallization processes where drying endpoints can be detected by Near IR. Other cases where PAT can be used is in crystallization processes specifically where specific polymorphs need to be controlled.

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15 METHOD DEVELOPMENT FOR BIOMOLECULES

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ABSTRACT

Various modes of high-performance liquid chromatography (HPLC) are often applied to the separation of large biomolecules, including reversed-phase liquid chromatography (RPLC), hydrophobic interaction chromatography (HIC), ion-exchange chromatography (IEC), and size-exclusion chromatography (SEC). Sample complexity not only makes these separations difficult but also renders HPLC method development an arduous task. At best, the bioanalyst must first use knowledge of the sample to decide the separation goals, and then a separation mode can be selected. Once the separation mode is chosen, specific method development steps are often little more than trial and error. While general HPLC method development guidelines are a useful starting place, additional characteristics that must be considered

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are presented. We discuss the varying nature of biomolecule sample properties and the role of solute hydrophobicity in achieving adequate liquid chromatography (LC) resolution in each of the common modes of LC. Emphasis is placed on the separation of peptides and proteins for purely analytical (rather than preparative) purposes.

I. INTRODUCTION

With the extensive growth of the biotechnology and natural product fields over the past decade, biomolecules are playing a larger role in pharmaceutical development. From lipid drug-delivery systems to gene therapy and carbohydrate-based drugs, the biological activity of large and complex compounds is being expressed and utilized. In order to use these molecules as active ingredients in pharmaceutical products, the purified compound must be available and method of analysis must be developed for accurate quantification of the component. In the United States, if a high-performance liquid chromatography (HPLC) method is used for quantification of a biomolecule with a new drug application (NDA) to the Food and Drug Administration (FDA), validation is required following the International Conference on Harmonization's (ICH) Q2A and Q2B guidelines.[1] Validation is required for all biomolecules including antibiotics, peptides, DNA products, polypeptides, and proteins. Acceptance criteria may be different for large biomolecules. For example, wider confidence levels may be appropriate and may be based on levels achieved during method development and validation. Impurity testing for biomolecules may not be required. Often biomolecule analysis is supported by several methods such as biological assay, along with an HPLC method. The ICH's Q6B specifically addresses specifications for large, complex biomolecules.

Liquid chromatography is already widely used in the pharmaceutical industry as the instrument for purification and quantification of many pharmaceutical compounds and preservatives due to its relative ease of use, the wide variety range of selectivity that can be chosen, and its quick analysis time.[2] These same advantages exist for using HPLC to analyze biomolecules. These biomolecules include proteins, peptides, carbohydrates, lipids, and nucleic acids. The large molecular weight and the presence of multiple functional groups on these biomolecules make their HPLC separation different from small molecules. For most of these molecules, several modes of HPLC can be used to achieve separation and purification. HPLC column and detector technology have grown, paralleling the need to detect biomolecules, leading to simpler method development options. This chapter describes how structure of biomolecules affects HPLC separation including successful employment of various HPLC modes to achieve these separations, and recent advances made in regard to analyzing biomolecules with HPLC. We assume that the reader possesses a basic knowledge of HPLC and method development

strategies. Characteristics unique to the HPLC of biomolecules will be discussed.

Several factors complicate the separation of biomolecules by HPLC. Protein mixtures, as representative biomolecule samples, represent a highly complex mixture. Drawing generalizations for protein samples does not recognize, for example, that some proteins may be insoluble solids, while others are liquids. Some proteins may comprise the major portion of a sample and others are trace components. "Purification" processes do not necessarily mean the isolation of a single compound and subtle differences between individual proteins must be recognized.[3] Next, their tertiary structure, which is not only related to biological activity, but can also mean that two or more compounds with similar molecular weight and molecular makeup may be present while quantification of only one (or each separately) is essential. The size of these compounds (molecular weights ranging from 100 to 10,000 or higher) may result in degradation products or related substances that can easily have a molecular weight difference of less than 1%. These issues mean that resolution must be exceptional. The final issue complicating HPLC separation is the functional groups that may be present. Some of those functional groups include amines, carbonyls, carboxylic acids, and others. These functional groups can participate in hydrogen bonding and type interactions leading to strong associations between the biomolecule and a pharmaceutical matrix or the biomolecule and the stationary phase. Protein solubility, ionic charge, size and shape, and surface features are factors that influence the choice of HPLC mode.

A. Proteins and Polypeptides

Amino acids are the basic units of polypeptides and proteins. Both are polymers of amino acids, with a peptide having less than 50 amino acids residues and a protein having more than 50 amino acid residues. Proteins generally will have a molecular weight greater than 5000 Da. The primary amino acid sequence and tertiary structure (i.e., three-dimensional shape) are the most important features to consider when planning an HPLC separation. Knowledge of the primary amino acid sequence allows for consideration of which functional groups are present in what quantity. Both amino acid side-chain and main-chain functional groups can interact with HPLC phases. Knowledge of the protein's tertiary structure provides consideration of which functional groups are on the exterior of the protein. With a protein in its native conformation, surface side chains will have the greatest interaction with the HPLC support.

During the course of an HPLC separation, unfolding or denaturation of a protein or peptide may occur. Irreversible unfolding will lead to a loss of the protein's biological activity. This may not be a problem for a quantitative assay, but for preparative isolation and purification

by HPLC, maintenance of biological activity may be essential. Unfolding can occur as the protein moves through the HPLC system, and during this unfolding, interior amino acid side chains will be exposed.[4] Often, the interior amino acids of a protein are predominantly hydrophobic and, during unfolding, additional interactions with non-polar stationary phases will occur, leading to increased retention and decreased separation efficiency. Unfolding can occur in different degrees, and the native conformation and unfolded conformations can have different retention times. Various components of the chromatographic system can contribute to protein unfolding, including stationary phase hydrophobicity, mobile phase polarity, mobile phase pH, ion-pairing agents, detergents, and oven temperature. The possibility of intermolecular interactions must be considered when unexpected peaks or low recovery occurs during analysis.

Proteins and peptides can be analyzed by several HPLC modes, with reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC) the most common and the easiest for method development. However, ion-exchange chromatography (IEC) and size-exclusion chromatography (SEC) are also used to successfully separate and purify mixtures containing proteins and/or peptides. A comparison of these modes demonstrates the general principles of each technique. Consider a mixture of peptides S2 through S5; each is divalent with the hydrophobicity increasing from S2 through S5. Figure 1 shows the differences between chromatographic modes.[5] In the upper RPLC chromatogram, elution is in order of increasing hydrophobicity. The addition of acetonitrile in the mobile phase of the cation exchange separation in the middle chromatogram induces hydrophilic interactions with the stationary phase (eliminating hydrophobic interactions), resulting in a reversal of elution order and deterioration of the quality of the separation. The final chromatogram is a mixed-mode HIC-IEC (cation exchange) separation. In this case, additional acetonitrile greatly promotes hydrophilic interactions and complementary ionic interactions.

B. Carbohydrates

Carbohydrates are classified as monosaccharides, disaccharides, oligosaccharides, and polysaccharides, depending on the number of sugar units making up the molecule. Oligosaccharides contain three to nine sugar units, and polysaccharides contain ten or more sugar units. Maltodextrins are an example of an oligosaccharide, while starch is a polysaccharide. Sugar alcohols are carbohydrate aldoses, where the aldehyde functionality is replaced with a primary alcohol.

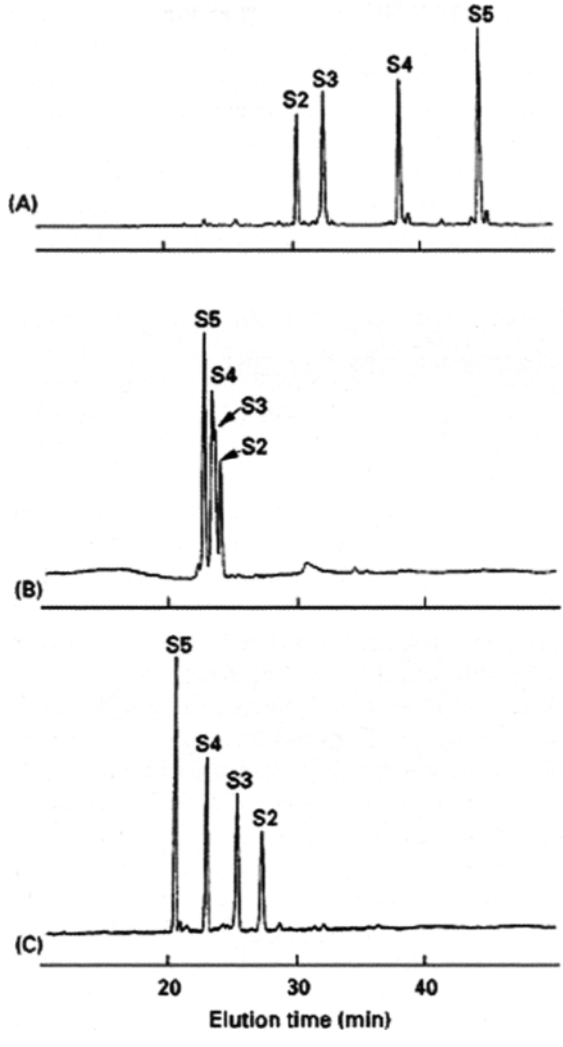

FIGURE 1 Comparison of HPLC modes for peptide separations: (A) RPLC, (B) IEC in the cation-exchange mode, and (C) mixed-mode HIC-IEC. RPLC was performed with a C_8 column and a linear AB gradient of 0.5% acetonitrile per minute at a flow rate of 1 mL/min, with eluent A = 20 mM aqueous triethylammonium phosphate (TEAP) at pH 3 and 100 mM $NaClO_4$ and eluent B = same as eluent A with 60% (v/v) acetonitile. IEC was performed with a polysulfoethyl strong cation exchange column and a linear AB gradient of 5 mM/min $NaClO_4$ following 5 min of isocratic elution with eluent A and eluent A = 20 mM aqueous TEAP at pH 3 and 10% (v/v) acetonitrile and eluent B = same as eluent A with 400 mM $NaClO_4$. HIC-IEC conditions were the same as IEC with 80% (v/v) acetonitrile in eluents A and B. (Reproduced with permission from reference 5.)

HPLC analysis of carbohydrates is complicated by the lack of chromophores that allow simple ultraviolet (UV) detection. The presence of numerous carbonyl or aldehyde functional groups means that hydrogen bonding between the carbohydrate and mobile phase, matrix components, or other carbohydrates may occur leading to incomplete separation. Many carbohydrate species are also easily oxidized and unless such a reaction is being utilized as part of the analysis, care should be taken to exclude oxidizing species from the HPLC system.[6]

II. PROTEIN AND PEPTIDE SEPARATIONS BY HPLC

A. Reversed-Phase Liquid Chromatography and Hydrophobic Interaction Chromatography

RPLC and HIC are popular means of separating or purifying proteins and peptides. While the hydrophobic character of proteins and peptides enables elution from RPLC and HIC columns, the mixed hydrophobicity and charge characteristics of protein mixtures lead to non-ideal chromatographic behavior. These HPLC methods tend to work well with small proteins and peptides, but suffer with larger or more unstable protein samples.[3] Large molecules (molecular weights may be greater than 20,000) have complex, three-dimensional structures and, as a result, RPLC is not as straight forward as for small molecules. Since the interior portion of many folded proteins has a high concentration of hydrophobic amino acid residues, any unfolding of the protein during analysis will lead to more hydrophobic surfaces being available to interact with the nonpolar ligates on the column. Globular proteins, in particular, can unfold as they move through the column. Any unfolding means the protein or peptide changes conformation and that different conformation will have a later retention time than the native structure. It is also possible that not all of the protein sample will undergo unfolding and the same protein will elute in two or more bands. The numerous opportunities for hydrophobic interactions in RPLC and HIC analyses make unfolding an important problem. Since unfolding may mean loss of biological activity, developing RPLC and HIC methods where biological activity is retained is even more complicated. The stationary phase support, ligates, surface tension, mobile phase polarity, temperature, pH, and mobile phase additives can all contribute to unfolding and may need to be adjusted during method optimization. Conformation changes due to unfolding are minimized at low temperatures and methods designed to prevent unfolding may be more successful at 25–30°C.[7] Some protein unfolding is reversible. Partial unfolding that may occur during RPLC or HIC analyses does not necessarily mean bioactivity is lost. Both chromatographic modes have been used successfully to isolate biologically active proteins.[2,7]

For an RPLC or HIC method, parameters to be optimized include column (stationary phase) choice, type and concentration of ion-pairing agent, organic solvent choice, pH, salt choice and concentration (HIC), mobile phase gradient, and temperature. The aspects of the column that are particularly important to protein or peptide analysis are pore size and particle size. For RPLC, sample loading capacity is related to the effective surface area available for interaction, a property related to the particle size of the packing, among other factors. Particle sizes of RPLC columns range from 1.5 to 25 μm. Band broadening can be a problem with large samples. HIC columns have particle sizes of 15–65 μm, and a larger sample can be accommodated, assuming other column factors including bonding density, type of bonding, porosity, and stationary phase volume are equal. For protein or peptide separation, an appropriate pore size can be chosen based on knowledge of molecular diameter. The minimum pore diameter that is acceptable for protein–ligate interaction is four times the protein's diameter. For beginning method development for a protein separation, choosing a pore size of greater than 30 nm will ensure acceptable diffusion of the large analyte into the pore. For peptide separation, a pore size of 10 nm is generally sufficient.[7]

The mobile phase characteristics for separating proteins and peptides by RPLC or HIC are similar to those employed during analysis of small molecules. In RPLC, this is a water-organic eluent. Typically, aqueous-based mobile phases are used since non-aqueous phases contribute to protein denaturation. Often the mobile phase is at a low pH to stabilize ionizable side chains. A gradient mobile phase program that increases the concentration of organic is utilized for samples requiring separation of several components. Gradient programs running to 100% organic are common for pharmaceutical products that have complex matrices in order to ensure elution of compounds that associate strongly with the stationary phase. The gradient used in HIC is an increasing salt concentration in a buffered aqueous mobile phase. For charged proteins, ion-pairing agents are generally used. Trifluoroacetic acid (TFA) is the anionic ion-pairing agent most often used. However, it has been shown that increasing reagent hydrophobicity (from phosphate < TFA < pentafluoropriopionic acid < heptafluorobutyric acid) has a pronounced effect on peptide resolution.[8] Efficiency increased with increased acid concentration.

B. Ion-Exchange Chromatography

IEC has found a home as the common technique for the separation of charged species. IEC achieves separation of proteins and peptides by analyte–ligate electrostatic interactions. Proteins and peptides may have multiple charged functional groups. Electrostatic interactions between

ions in the solute, mobile phase, and stationary phase are the primary contributors to retention. Elution of these biomolecules is complicated by the presence of multiple charged regions on a molecule. In singly charged molecules, elution is linear in response to pH change. Elution becomes nonlinear with pH where multiple interactions can occur.[2] The tertiary structure of proteins should also be considered during an IEC separation. Surface or surface accessible ionizable groups will participate in retention. Those groups, but not necessarily all ionizable groups, participate in solute–ligate interactions. Possible repulsive forces occurring between similar charges in the solute and stationary phase may further complicate charge-type interactions.

Method development parameters in IEC include stationary phase choice, mobile phase pH, ionic strength, choice of mobile phase ions, and temperature. The IEC column consists of the stationary phase resin with immobilized strong or weak cation or anion exchangers. The degree of ionization differentiates a strong exchanger from a weak exchanger. Strong anion exchangers, for example, quaternary amine functional groups, or strong cation exchangers, such as sulfonated functional groups, are completely ionized. Their charge density, or ability to effect an exchange, does not change with a change in pH. Separations of proteins and peptides employing strong exchangers are more straightforward. The exchange capacity of weak anion exchangers, such as primary, secondary, or tertiary amines, or weak cation exchangers, carboxylic acids, is variable. The degree of ionization of weak exchangers changes as mobile phase pH approaches the pK_a of the exchanger's functional group. The retention mechanism for IEC of proteins and peptides becomes very complicated with the use of weak exchangers. As a basic guide for choosing a column, acidic proteins are analyzed on an anion exchange column, and basic proteins are analyzed on a cation exchange column.[7] Most native proteins are negatively charged; consequently, anion exchange columns are used.

Elution in IEC occurs through increasing the ionic strength of the mobile phase. Thus, a gradient of increasing salt concentration is the minimum requirement for an IEC mobile phase. The gradient should increase the salt concentration very gradually. A small change in ionic strength can have a large effect on retention since many groups on a protein may be affected at one time. The choice of salt affects selectivity, although predicting the exact effect of any particular salt is difficult.[9] The pH of the mobile phase may be adjusted to change the charge on the protein or peptide. Such an adjustment may be useful for increasing or decreasing retention time. Changes in pH may also affect selectivity if there are areas of concentrated charge on one or more of the proteins being separated. Small amounts of organic modifier may be added to minimize hydrophobic interactions with the stationary phase.

C. Size-Exclusion Chromatography

SEC tends to be used for separation of high molecular weight species from biological matrices where non-protein contaminants or undesirable protein aggregates are present. It does not play an extensive role in identification or quantitation of proteins or peptides mainly because of the low resolution. So, SEC has limited usefulness in regards to biomolecules.

In simplest terms, SEC separates based on molecular size. Size, however, as it pertains to proteins and peptides with tertiary structure, comes from molecular hydrodynamic volume. The hydrodynamic volume relates to the hydrated molecular radius. The aqueous nature of SEC mobile phases results in polar interactions and water molecules may associate with the proteins and peptides. This association gives the molecule an apparently larger molecular size. Proteins can bind multiple water molecules and this binding does not occur linearly with increasing molecular weight. Proteins can have different degrees of hydration. Thus, predicting elution order based solely on molecular weight becomes difficult. The variety of functional groups on proteins means that hydrogen bonding, dipole, hydrophobic, and charge transfer interactions can take place between the proteins and other small molecules in the mobile phase. These small molecules could be from mobile phase additives or excipients in a pharmaceutical formulation. This association with small molecules causes a change in the molecular hydrodynamic volume, in addition to volume changes due to hydration. All changes in hydrodynamic volume affect SEC retention behavior. Any interaction between the solute and stationary phase, especially hydrophobic interactions, can lead to a protein unfolding as the protein moves through the column. Unfolding of globular proteins can also lead to changes in hydrodynamic volume. Unfolding can alter retention time. For example, protein aggregation can lead to decreased SEC retention time.

Method development parameters for SEC include buffer choice, salt concentration, organic modifier, and flow rate. SEC columns, especially those advertised for protein separation, have been developed such that the non-size exclusion interactions are minimized. Hydrophobic or charge-transfer interactions between the protein and support lead to partitioning or ion-exchange behavior. Most commercial supports will be neutral and hydrophilic to prevent those interactions from occurring. Inorganic oxide packings must be surface modified to cover the surface silanol groups with a neutral molecule. Often these packings cannot be used in a basic (pH $>$ 8) environment. Organic packings are more susceptible to protein adsorbance due to the slight hydrophobic character of these columns. Columns with organic packings may degrade under high pressures, limiting the flow rate that can be implemented in the method. However, organic packings are more pH resistant and can be used at high pH.[4]

Mobile phase optimization is the most difficult portion of SEC method development. SEC mobile phases are generally neutral buffers with a salt concentration near 0.1 M. However, method development will likely require adjustment of pH and salt concentration. The wide variety of possible protein and peptide combinations makes it difficult to suggest particular changes. Not only does the hydrodynamic volume of proteins change with pH, mobile phase additives, or organic modifiers, but for peptide separation, a significantly different mobile phase composition may be required to achieve elution of different classes (e.g., acids, neutrals, etc.).[4]

D. Protein and Peptide Detection

UV detection is a very popular detection method for pharmaceuticals. The spectroscopic method has become even more popular as more publications discuss the uses of photodiode array (PDA) detection. Peptide and protein detection can occur at 210–220 nm due to the absorbance by the peptide bond. UV spectra of proteins will often have a large peak around 210 nm, and a smaller peak around 280 nm. The aromatic amino acid side chains absorb at 280 nm. Simple single wavelength UV detection is limited to being a weighted average of all species present. PDA detection can offer increased sensitivity and multiple wavelength analysis (which allows for peak purity to be assessed). The prominence of computer-assisted analysis in pharmaceutical labs has led to derivative spectroscopy as a detection technique. First, second, third, even fourth derivatives of the UV spectra can be plotted. The resulting new minima or maxima offer additional information regarding the peak. Fluorescence and electrochemical detection are also used for protein and peptide detection. The evaporative light scattering detector (ELSD) is also beginning to be utilized in pharmaceutical assays, although it has certain limitations with pharmaceutical matrices. The ELSD is likely to find usefulness for methods with highly volatile mobile phases and lower-volatility high melting point analytes.

III. CASE STUDIES

The preceding discussion of HPLC method development describes the important chromatographic parameters and their relationship with structural properties of biomolecules. These relationships can be observed by example demonstrated in the following case studies.

A. Columns and the Separation of Proteins and Peptides

An investigation of HPLC protein and peptide separations using micro-bore HPLC columns was carried out by Issaq et al.[10] The group

looked at the effect of changes in column parameters and mobile phase composition on separation efficiency and selectivity. They also looked at optimizing HPLC conditions for electrospray ionization-mass spectrometric detection (ESI-MS).

Many columns can be used to achieve protein or peptide separation, including newer column technologies such as polystyrene/divinylbenzene (PS/DVB) polymeric stationary phases and monolithic supports. The group found improved sensitivity using smaller diameter columns and the smallest pore size that is appropriate for the size of protein or peptide being analyzed. A tryptic digest of myoglobin showed maximum sensitivity with a 0.18 mm i.d. column and silica pore size of 90 Å. A 300 Å pore size would be appropriate for analysis of larger proteins and polypeptides. Several bonded phases, C_{18}, C_8, C_3, and CN, were tested for their effect on a polypeptide separation. The polypeptides separated include Rnase, insulin, cytochrome C, lysozyme, pavalbumin, CDR, myoglobin, carbonic anhydrase, S-100b, and S-100A. Increasing the stationary phase alkyl chain length from C_3–C_8 to C_{18} increased retention and resolution for all of the polypeptides except CDR and myoglobin. Those two analytes nearly co-eluted on the C_{18} column, co-eluted on the C_8 column, then reversed elution order and eluted well resolved on both the C_3 and CN columns. Thus, the increased hydrophobicity of a longer alkyl chain can lead to changes in the three-dimensional shape of a protein that may account for the change in selectivity.

B. Mobile Phase Conditions and the Separation of Proteins and Peptides

For the investigation of mobile phase conditions in the same study,[10] a reversed-phase gradient was used since the majority of protein and peptide separations are carried out using RPLC. The basic gradient system was made of aqueous +0.1% TFA mobile phase A and organic +0.1% TFA mobile phase B with a linear gradient increasing in organic content. There was little difference in separation when acetonitrile and methanol were compared as the organic modifier. The only difference in the separation of a mixture of five peptides (Gly-Tyr, Val-Tyr-Val, methionine enkephalin, leucine enkephalin, and angiotensin II) was improved resolution of the leucine enkephalin and angiotensin II peaks in the chromatogram with methanol as the modifier. As the final concentration of organic in the linear gradient was increased (5–35%, 5–50%, 5–75%), retention decreased and efficiency increased. Selectivity changes occurred between leucine enkephalin and angiotensin II. At 5–35% acetonitrile, the peaks were resolved; at 5–50%, the peaks co-eluted; and at 5–75%, the peaks were resolved but the elution order was reversed. Several ion-pairing agents were compared including TFA, heptafluorobutyric acid (HFBA), and formic acid. In the separation of a cytochrome C digest, TFA gave the best sensitivity, peak shape, and resolution. The effect of ion-pair

agent concentration was also studied using the five-peptide mixture. A change in concentration of TFA affected peak height, selectivity, and resolution. An increase in TFA concentration improved peak symmetry and peak width. The changes in resolution and selectivity did not follow a trend.

C. Ion-Pairing Agents and the Separation of Peptides

A detailed series of studies on ion-pairing agents and HPLC peptide separations was carried out by Hodges and coworkers.[8,11–13] They looked at the concentration of TFA and determined that increasing the concentration of TFA to 0.20–0.25% increased sensitivity and separation of a mixture of peptides with similar charges.[11] The group also found that increasing the temperature from 25 to 70°C gave additional improvement in separation. The same group compared four ion-pairing reagents (phosphoric acid, TFA, pentafluoropropionic acid (PFPA), and HFBA) at various concentrations for the effect on separating two peptide mixtures, one mixture containing peptides with the same net charge, the other mixture containing peptides with varying net charge.[8,12] The researchers found that for both peptide mixtures, retention time increased with increasing concentration and hydrophobicity of the reagent. Hydrophobicity had a larger affect on change in retention than concentration change. Increasing hydrophobicity of ion-pairing reagent also improved peak shape. For peptides of identical charge, resolution was not affected by choice of ion-pairing reagent and was affected only slightly by an increase in concentration. For peptides of varying charge, resolution increased with increased reagent concentration, although improvement in resolution diminished between 20 and 40 mM for all reagents. At 20 mM, the best overall resolution was achieved with phosphoric acid, although phosphoric acid also showed the least change in resolution with an increase in reagent concentration. Selectivity changes occurred with a change in reagent. The researchers also found that addition of a salt, such as sodium perchlorate ($NaClO_4$), to the mobile phase increased retention time and could improve peak shape.[13]

D. The Separation and Evaporative Light Scattering Detection of Phospholipids

Two research groups[14,15] recently published methods to separate and quantitate phospholipids using HPLC with ELSD. Both methods separated a variety of phospholipids including phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), sphingomylelin (SM), and phosphatidic acid (PA).

Rombaut et al.[14] utilized a 3.2 × 150 mm, 3 μm silica column and a linear mobile phase gradient. The gradient began with 87.5:12:0.5 chloroform:methanol:buffer (1 M formic acid with triethylamine to pH 3) adjusting to 28:60:12 in 16 min. The buffer pH was found to affect peak shape and resolution of PS, PI, and PC. The acidic pH also increased column life. Several ELSD conditions were critical for detection of phospholipids. The optimal flow rate of the nebulizing gas (N_2) was 1.4 mL/min and optimal nebulizing temperature was 85°C. Increasing flow rate led to decreased response of all phospholipids. Increasing temperature had a similar but less pronounced effect. Detection limits for the phospholipids ranged from 11 to 40 ng.

Zhang et al.[15] used a 4.6 × 250 mm, 5 μm silica column with a gradient flow using a three-part mobile phase. An initial, isocratic composition of 42:5:53 isopropanol:water:hexane at flow rate of 0.8 mL/min was held for 8 min, then programmed to a final mobile phase composition of 54:10:36 from 8 to 25 min at 1.2 mL/min. Finally, an isocratic mobile phase composition of 66:17:17 was set from 25 to 35 min at 0.8 mL/min. The percent water in the mobile phase was the critical factor leading to elution and resolution of the phospholipids. ELSD conditions were 2 mL/min carrier gas (N_2), drift tube temperature of 63.5°C, and pressure of 0.4 MPa. This group determined that the drift tube temperature was the most important ELSD parameter during the analysis. Temperatures lower than 63.5°C led to baseline drift, and temperatures above 63.5°C led to decreased signal-to-noise ratio.

IV. SUMMARY AND CONCLUSIONS

Prior to beginning the HPLC method development process, biomolecular structure and complexity is considered in choosing the chromatographic mode. It must be kept in mind that parameters like stationary phase hydrophobicity, mobile phase polarity and pH, ion-pairing agents, and temperature can play vital roles, not only in the separation process, but also in deleterious protein denaturation or other structural disruption. In RPLC and HIC, stationary phase and mobile phase selection, stationary phase support particle size, ion-pairing identity and concentration, organic solvent selection, pH, temperature, and gradient conditions are all important method development parameters. For IEC, separations are accomplished due to analyte–ligate interactions and factors such as stationary phase, mobile phase pH, ionic strength, counterion selection, and temperature must be considered for method development. Gradient elution with increasing mobile phase ionic strength dominates IEC separations. In SEC, buffer choice, pH, organic modifier, and flow rate are primary method development considerations.

ABBREVIATIONS

ELSD	Evaporative light scattering detection.
ESI-MS	Electrospray ionization-mass spectrometry.
FDA	Food and Drug Adminstration.
HFBA	Heptafluorobutyric acid.
HIC	Hydrophobic interaction chromatography.
HPLC	High-performance liquid chromatography.
ICH	International Conference on Harmonization.
IEC	Ion-exchange chromatography.
LPC	Lysophosphatidylcholine.
NDA	New drug application.
PA	Phophatidic acid.
PC	Phosphatidylcholine.
PDA	Photodiode array.
PE	Phosphatidylethanolamine.
PFPA	Pentafluoropropionic acid.
PI	Phosphatidylinositol.
PS/DVB	Polystyrene/divinylbenzene.
RPLC	Reversed-phase liquid chromatography.
SEC	Size-exclusion chromatography.
SM	Sphingomylelin.
TFA	Trifluoroacetic acid.
UV	Ultraviolet.

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METHOD VALIDATION

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ABSTRACT

Method validation is supposed to be the finishing touch on a proper method development process. The critical performance indicators of the method need to be investigated thoroughly during the development phase of the method. At validation, there should be no surprises toward failures on the method performance. Usually method validation is covered by standard procedure approaches and is executed within the shortest possible time in a

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GMP compliance environment. The results are documented in the validation report summarizing the obtained validation results.

I. INTRODUCTION

Validation is the process of collecting documented evidence that the method performs according to the intended purpose.[1–5] The validation characteristics and the acceptance criteria to be applied in validation of HPLC methods for MAA/NDA filings and marketed products should comply with the international guidelines on method validation.[1,4,6] "*Validation is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended application*".[4] The choice of characteristics to be evaluated depends on the purpose of the analytical method and the product development stage that the analytical method is supporting. It is recognized that methods are often evolutionary and that methods in early development will generally require less extensive validation than methods in final development. Validated analytical methods should be used for the analysis of drug substance and drug product. Appropriate validation of analytical methods for other materials (e.g. intermediates, excipients, preservatives, etc.) used in the production of drug substances and drug products should also be considered. For a smooth performance, it is important that the analytical method clearly documents the details of performing the analysis. It should describe in detail the steps necessary to perform the analytical test. This may include, but is not limited to, the principle of the test method, the preparation of samples, reference standards, and any reagents or solutions, the use and required functional specifications of the apparatus, the generation of the calibration curve, system suitability tests (SSTs), and the use of the formulae for the calculations.[4] According to the ICH,[2,3] the following types of tests may be considered:

- Identification tests: intended to ensure the identity of an analyte in a sample.
- Quantitative tests for impurities' content: intended to accurately and quantitatively reflect the purity of a sample.
- Limit tests for the control of impurities: intended to accurately reflect the purity characteristics of a sample as a limit test.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product: intended to accurately and quantitatively measure the analyte (drug substance or preservative) present in a sample.

These methods may also include analysis for potency or content uniformity, and the measurement of analyte in dissolution samples. From the perspective of the validation approach to be applied, specifically

toward HPLC methods, it would be more practical to describe the following type of tests:

- Type 1: assay and identification of the main component(s) (active, preservatives and key excipients).
- Type 2: assay and identification of the impurities.
- Type 3: limit tests.

The recommended validation characteristics to be performed for each of the different types of tests are described in Table 1. There are validation characteristics, which are considered mandatory for regulatory guidances and others that are necessary because of good quality practice.

TABLE 1 Recommended Validation Performance Characteristics According to the Type of Analytical Test Method

Test type	Type 1	Type 2	Type 3
Validation characteristics mandatory			
Specificity[a,b]	+	+	+
Accuracy	+	+	–
Precision			
System repeatability	+	+	–
Analysis repeatability	+	+	–
Intermediate precision[c]	+	+	–
Reproducibility	+	+	–
Detection limit	–	–	+
Quantitation limit	–	+	–
Linearity	+	+	–
Range	+	+	–
Robustness[d]	+	+	+
Additional validation characteristics			
Stability of solutions	+	+	+
Reporting threshold	–	+	–[e]
Filtration study	+	+	+
Relative response factor	–	+	–
System suitability tests	+	+	+
Method comparison	+	+	+

–, Performance characteristic not evaluated; +, Performance characteristic is evaluated.
[a]A combination of two or more analytical procedures can compensate for the lack of specificity for an individual identity test.
[b]Lack of specificity for an assay used for release may be compensated by testing for impurities.
[c]Intermediate precision may not be necessary if reproducibility has been evaluated.
[d]Method robustness may be evaluated during method development.
[e]May be needed in some cases.

Scientifically sound rationales should be provided for characteristics that are not investigated and validated.

II. VALIDATION PROCESS

Prior to starting any practical work, a written validation protocol should be established describing at least the different validation characteristics to be evaluated with the expected acceptance criteria, reference to or description of the analytical test method, and how the validation will be performed. After reviewing and approving the protocol, practical experiments are carried out as described.[7] During the practical work, unexpected incidents are bound to occur. An established procedure should be followed that describes the handling of incidents during method validation. Any problem or failure should be investigated and well documented. After finalizing the practical work, a validation report should be prepared that summarizes all the results obtained. Individual values as well as summary tables, linearity plots, together with representative chromatograms should be provided. Any deviations observed should be commented on and the appropriate conclusions proving that the method is suitable for its intended use are drawn.[4,7]

III. VALIDATION CHARACTERISTICS

The validation characteristics required by current regulatory guidelines to be examined during validation are defined in this section. Where appropriate, a discussion is provided together with typical acceptance criteria for the different validation characteristics.

A. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradation products, matrix, etc. The lack of specificity of an individual analytical method may be compensated for by an additional analytical method.[2,3] Specificity acceptance criteria comprise many quality aspects of the chromatographic separation.

Blank peaks are preferably absent or if present should not interfere with other peaks of interest. Placebo peaks in drug product methods should be separated from other relevant peaks. Unspecified degradation products and impurities should be separated from the main component(s), and from other specified degradation products for drug product methods. Synthesis impurities are not specified in drug product methods, but are controlled by the drug substance methods.[8,9] In drug substance

methods, the unspecified degradation products and impurities should therefore also be separated from the specified impurities. Specified impurities should be separated from the main component(s), from the specified degradation products, and from each other for drug substance (DS) methods, and preferably from each other in drug product (DP) methods. Specified degradation products should be separated from the main component(s), from the specified impurities and from each other.

Peak purity of the main components should be assessed preferentially by an orthogonal technique and should not result in new impurities above the reporting threshold (RT) level. The orthogonal technique can be either another chromatographic method or a specific spectral analysis identification method like, for example, UV-photo diode array detection or MS detection. When peak purity of the main components is assessed with LC-UV-DAD, the UV-spectra in the front, middle, and the tail of the main compound peak should be comparable. Peak purity of the main components may also be performed with LC-MS. Similarly, the MS-spectra in the front, middle, and the tail of the main compound peak should be comparable. Additionally, the impurity profile using an orthogonal chromatographic technique/method should be comparable and may not result in new impurities above the RT level.

The identity of all impurities and degradation products should be confirmed by spiking experiments using certified reference material of synthesized impurities, or using LC-MS or LC-DAD on impurities in representative sample batches. Matching retention time windows, relative retention time windows, or UV- and MS-spectra confirm the identity of all compounds. If the impurities or degradation products are unavailable, specificity may be demonstrated by comparing the test results to a second well-characterized procedure. In addition, stress studies should demonstrate that impurities and degradants from the active pharmaceutical ingredient and drug product excipients do not interfere with the quantitation of the main product.

When criteria for specificity are not met this often indicates that the method is not sufficiently developed. As a consequence, it is likely that the criteria for accuracy, precision, and linearity may also not be fulfilled.[1] *Lack of specificity may be compensated by other supporting analytical methods but finally the test method(s) should be able to ensure the identity of an analyte (identification test method), ensure that the method allows an accurate statement of the content of impurities (purity test methods), provide an exact result, which allows an accurate statement on the content or potency of the analyte in the sample (assay test methods).*[2]

B. Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the obtained (by practical experiments) value and the

value that is accepted either as a conventional true value or as an accepted reference value. Accuracy is expressed as the % recovery. Typically, it is requested to test three replicates per level at a minimum of three concentration levels across the specified experimental range. The acceptance criteria are set for the assay range of the main compound and for the range of the impurities. For the assay range of DS methods (80.0–120.0%), this may be between 98.5 and 101.5% for the average recovery value and between 98.0 and 102.0% for each individually obtained recovery value. For the assay range of DP methods (80.0–120.0%), this may be between 98.0 and 102.0% for the average recovery value and between 97.0 and 103.0% for each individually obtained recovery value.

For the impurities in both DS and DP methods, the acceptance criteria for accuracy may range from 50.0–150.0% average recovery at the RT level to 80.0–120.0% average recovery at the specification and maximum levels within the concentration range.

C. Precision

The precision of an analytical method *expresses the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample under the prescribed conditions in the test method.* Precision is usually determined at different levels: repeatability (system repeatability, analysis repeatability), intermediate precision, and reproducibility.[3,4,10,11]

The term precision describes the distribution of the individual results around their average. Frequently, it is confused with the term accuracy, which refers to the difference between the mean of the results (e.g. in recovery experiments) and the "true" value.[11,12] The standard deviation, relative standard deviation (coefficient of variation), and confidence interval should be reported for each type of precision investigated[3] in addition to the individual results obtained and the mean.[4]

1. Repeatability

(a) System Repeatability:

The system repeatability is measured by multiple injections of the same reference solution and expressed as the relative standard deviation on the measured peak areas. The system precision considers the lowest variation of the analytical system, while analysis repeatability is related to all aspects of the test method including sample preparation.[1,11] The information is provided as part of the validation data and as a SST.[10] Better precision of the test method results in a better control of the test substance, since more confidence can be placed into the obtained result.[13] Including system repeatability as SST in the method is an essential part of the test method. However, the acceptance criterion should be related to the specification limit of the product.

The RSD on the obtained responses for a selected compound (usually the main compound) is used as a measure and should typically be ≤2.0%* for drug product and ≤0.73% for drug substance methods for a response at 100% concentration level. At a lower concentration level (range of the impurities), the RSD on the obtained response of the selected compound is generally limited to ≤10.0% for drug product and drug substance methods.

(b) Analysis Repeatability:

The analysis repeatability (within day or intra-assay precision) is determined by analyzing a representative sample batch at the nominal sample concentration six times or with a minimum of nine determinations covering the specified range of the test method.[3]

Analysis repeatability expresses the precision of an analytical method under the same operating conditions over a short interval of time, e.g. the precision of the results of multiple sample preparations. The acceptance criteria are set for RSD on the average recovery of the assay range of the main compound and for the range of the impurities. For the assay range (80.0–120.0%), this may be ≤1.5% for DS methods and ≤2.0% for DP methods, calculated for the average. For the impurities, the acceptance criteria for the RSD on the average recovery may range from ≤25.0% at the RT level to ≤5.0% at the upper (maximum) concentration range level.

2. Intermediate Precision

Intermediate precision expresses within laboratory variations (effects of random events on the precision of the analytical method), e.g. different days, different analysts, different equipment, etc. If data to support reproducibility are available, it is not necessary to perform an intermediate precision assessment. The way to perform the test can be quite different. An ANOVA design approach is recommended, but generally the test is performed by multiple analyses ($n = 6$) of the same sample batch by two analysts on two different days, using independently prepared reagents and sample preparations, and preferentially performed on two different equipment set-ups. The pooled RSD is calculated for the two sets of data obtained by the analysts (e.g. $n = 6$ for each).

Acceptance criteria are set for pooled RSD (for comparable variances) on the average assay result for the analysis of the main compound and for the range of the impurities. For the assay range (80.0–120.0%), this may be ≤2.0% for DS methods and ≤3.0% for DP methods. For the impurities, the acceptance criteria for the pooled RSD on the average impurity levels may range from ≤30.0% at the RT level to ≤10.0% at the upper (maximum) concentration range level.

Additionally, the absolute difference in % between the mean assay values of the analysts is calculated and should be within ≤2.0% for DS

*A 2.0% limit is required by the guidelines, however, considering the current status of HPLC injection systems a limit of ≤1.0% would have been more appropriate.

methods and ≤3.0% for DP methods. For the impurities, the relative difference in % is calculated for the average impurity levels. This may range from ≤60.0% at the RT level to ≤20.0% at the upper (maximum) concentration range level.

3. Reproducibility

Reproducibility expresses the precision between laboratories. If data to support reproducibility are available, intermediate precision assessment is not required. Reproducibility is usually tested during the method transfer activity. The acceptance criteria for reproducibility are implicitly broader than that for intermediate precision. Typically, a factor of 2 is applied to the analysis precision criterion to obtain the acceptance criteria for reproducibility.

D. Linearity

The linearity of an analytical method is its ability (within a given range) *to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.* In some cases, the test data may have to be subjected to a mathematical transformation prior to the regression analysis. It is required to have at least five concentration levels (data points) to determine linearity. While scientifically ill-advised, correlation coefficients are generally applied as criteria for linearity, even in the ICH guidelines. According to the guidance documents, the correlation coefficient, *y*-intercept, slope of the regression line, and residual sum of squares should be submitted next to a plot of the linearity curves.[3]

Proposals for possible correlation coefficients limits may be for impurities in the lower range ≥0.99, for the main compound in the assay range (80–120%) ≥0.999, and for the main compound over the whole range (RT up to 120%) ≥0.99. More meaningful criteria are the RSD of the response factors and a visual check of the calibration line. (Response factor is the ratio of the detector response over the concentration.) Proposals of possible limits for the RSD on the response factors may be for impurities in the lower range ≤10.0%, for the main compound in the assay range (80–120%) ≤3.0%, and for the main compound over the whole range (RT up to 120%) ≤10.0%. The limit for the visual check of the calibration line is that a linear relationship is clearly demonstrated.

E. Range

The range of an analytical method is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical method has a suitable level of precision, accuracy, and linearity.

When linearity, accuracy, and precision criteria are fulfilled in the 80.0–120.0% range for the main component, the assay range is confirmed. For specified related impurities, the range is confirmed when linearity, accuracy, and precision criteria are fulfilled within the specified ranges of each impurity.

F. Quantitation Limit and Detection Limit

The quantitation limit (QL) is the lowest amount of analyte in a sample, which can be quantitatively determined with a suitable level of precision and accuracy.[2,4] The detection limit (DL) of an individual analytical method is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The DL and QL can be calculated in several ways,[3] e.g. from the signal-to-noise ratio.

As shown in Figure 1, the variance of the analytical signal increases with the decrease in the concentration level to be determined. Typically, variance of the analytical signal changes with time, instrumentation, analyst, and environmental conditions (e.g. temperature), etc. Therefore, it is difficult to set fixed limits for the DL and QL, but it is a good laboratory practice to make a rough estimation of the DL during method development by, for example, testing the performance on different instruments that are typically applied in the different labs where the method will be transferred.[14] The RT level, in contrast, is a fixed limit that is determined per analyte and per method, depending on its daily intake. For this reason, it should be targeted to develop methods with QL levels well below the RT levels. The QL should be subsequently validated by the analysis of a suitable number of samples near or at the QL. Since very

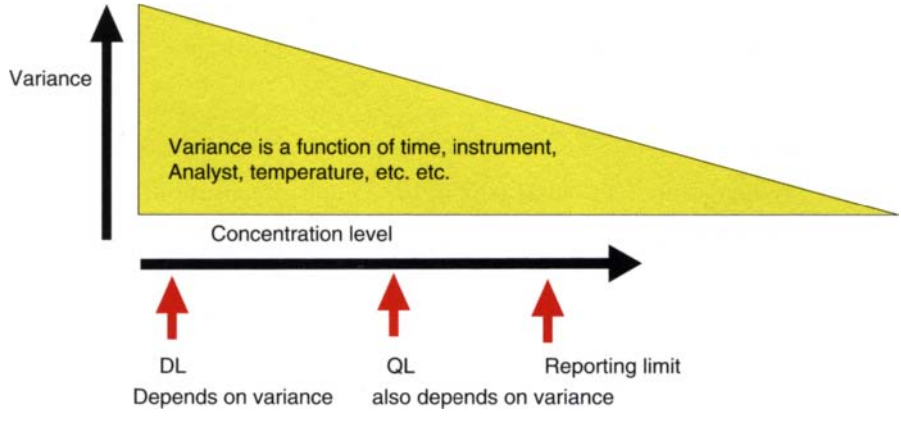

FIGURE 1 The DL and QL inversely vary with the concentration level. The reporting limit is a fixed level per analyte and per method.

low concentrations for the QL can be obtained, it makes more sense to perform this accuracy and precision study at the RT level from a practical point of view. Typical acceptance criteria for QL are a mean recovery at this level between 50.0 and 150.0%, with a RSD limit of ≤25.0%.

IV. ADDITIONAL VALIDATION CHARACTERISTICS

A. Stability of Solutions

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the analytical test method. An example of such a typical variation is the stability of the analytical sample solutions.[3] The stability is tested by determining the period of time over which the analytes of interest remain stable at specified storage conditions.

Proving the stability of solutions guarantees that representative results for the test sample will be reported and gives QC labs the opportunity to plan their analytical work in an efficient way. Stability of solutions can be proven for different types of solutions: sample solution (filtered and un-filtered), reference solution, selectivity solution, mobile phase, etc., each with their own acceptance criteria. The conditions of storage and the stability time period should be described unambiguously in the method description.

The acceptance criteria are based on relative differences between the initial value and the value at the specified solution stability time. For the main compound, this relative difference should for example be ≤2.0%. For the impurities, the relative differences may range from ≤50.0% at the RT level to ≤10.0% at the upper (maximum) concentration range level. Additionally, there should be no new degradation compounds higher than the RT detected.

B. Filtration Study

When a filter is used as part of the sample preparation, this study is set up to investigate the filtration process (e.g. the binding of the analytes of interest to the applied filtration device, filtration discard volume, the extraction of contaminants, etc.). Filtered and unfiltered results are compared for the main compound and the impurities. Binding of the analyte to the applied filter can lead to low recoveries of the assay and the use of organic solvents can extract contaminants out of the filter binding. This can be demonstrated by an overlay of a filtered and un-filtered blank solution. During method development, the filtration discard volume must be defined and described within the test method. All the accuracy

samples will be filtered in accordance to the method description and their recovery calculated against an unfiltered reference solution. If the accuracy test passes its acceptance criteria, the filtration can be accepted.

C. Relative Response Factor

A response factor is the ratio of the response of a component divided by its concentration. The relative response factor (RRF) of a component is the ratio of its response factor to the response factor of a reference component under the same operational conditions. The RRF is calculated and reported. For impurities for which no authentic reference material is available and for unknown peaks, the RRF is assumed to be 1.00.

D. Reporting Threshold

The RT is a limit above which ($\geq$) an impurity needs to be reported. The RT is determined as is described by the ICH guidelines,[8,9] and is generally 0.05% for DS and 0.10% for DP methods. Any impurity above this level should be reported. Especially for stability indicating methods, it may be useful to apply 0.03% as the RT, which will allow a better trending process for the degradation product levels below 0.05% and 0.10% for DS and DP methods, respectively.

E. System Suitability Tests

SST characteristics and limits are recommended as a component of any analytical method. It ensures that both methodology and instrumentation are performing within expectations prior to the analysis of test samples. SSTs have to be defined based on the practical experience obtained during method development[1] and/or the worst-case conditions of the robustness testing[1,15] and are related to the type of analytical method and the specification limits that this method is supporting. Measuring repeatability of injection has no added value for a method using area percent calculations while the acceptance criteria for system precision for a drug product method (95–105%) have to be less stringent than for a drug substance method (98–102%). During method validation, the SSTs will be performed prior to the validation analysis and evaluated for setting limits for subsequent testing.

F. Method Comparison

A method comparison study is performed to demonstrate that a new method is adequate for its intended use by comparing its analyses results

with the previously existing methods. It is expected that the newly developed final method is better than the previous methods. Differences in analysis performance should be evaluated according to sound scientific judgment.

V. ROBUSTNESS TESTING

A. Introduction

A method is considered to be robust when the effect of typical fluctuations in the method parameters on the outcome of the procedure is demonstrated to be negligible. Robustness is demonstrated by a test in which the effect of deliberate changes in the method parameters is studied on a number of method responses. The extent of the changes should reflect the normal variation in the method parameters that is observed during application in different labs. In general, the purpose of robustness testing is to indicate factors that can significantly influence the outcome of the studied responses.[1,16,17] This gives an idea of the potential problems that might occur when the method is repeated in different laboratories (transfer). In this way, potential problems can be anticipated by controlling the critical factors adequately, for example, by including a "precautionary statement" (ICH)[2,3] in the method description stating the accurate limits for the critical factors. However, the proposed limits should be feasible or redevelopment of the method should be considered. Additionally, it is possible to use robustness testing to determine system suitability limits for the system suitability parameters, a procedure that is recommended by the ICH.

Robustness testing of the chromatographic and the sample preparation factors is conducted either by an experimental design or a step-by-step approach (variables are evaluated sequentially). An experimental design approach is preferred since information is obtained in the shortest possible time, from a limited number of experiments. The choice of the experimental design requires a decision as to which factors and responses will be investigated and determination of whether main effects of the factors only or also the interactions will be examined. Typically, a Plackett Burman design (Table 2) will be sufficient.[16–19] The outcome of the robustness test is the identification of critical parameters and the determination of SST limits.

B. Chromatographic Method Parameters

A tentative list of factors that may be investigated in the robustness test is presented in Table 3. This list is not complete and additional factors may be added. The limits for the factor levels are proposals and

TABLE 2 A Plackett Burman Design in Which 8 Factors are Studied Simultaneously in 12 Experiments

Experiment	Dummy1	Column	pH	Temperature	Gradient_ start	Dummy2	Gradient_ end	Flow	Wavelength	Buffer_ concent- ration	Dummy3
Start	0	0	0	0	0	0	0	0	0	0	0
1	1	1	1	−1	1	1	−1	1	−1	−1	−1
2	1	1	−1	1	−1	−1	−1	1	1	1	−1
3	1	−1	1	1	−1	1	−1	−1	−1	1	1
4	1	−1	−1	−1	1	1	1	−1	1	1	−1
5	1	−1	1	−1	−1	−1	1	1	1	−1	1
6	−1	1	1	1	−1	1	1	−1	1	−1	−1
7	−1	1	−1	−1	−1	1	1	1	−1	1	1
8	−1	−1	−1	1	1	1	−1	1	1	−1	1
9	−1	−1	1	1	1	−1	1	1	−1	1	−1
10	−1	1	1	−1	1	−1	−1	−1	1	1	1
11	1	1	−1	1	1	−1	1	−1	−1	−1	1
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1
End	0	0	0	0	0	0	0	0	0	0	0

TABLE 3 Chromatographic Factors Potentially Investigated in a Robustness Study

	Factor	Units	Limits of factor levels
1	Column	Different lots	Preferentially two different lots of which at least one is new
2	Column temperature	°C	±5 absolute
3	pH of the buffer	Units	±0.2 absolute
4	Percentage organic modifier in the mobile phase at the start of the gradient or isocratic conditions	%	±1% absolute
5	Slope of the gradient	%/min	±5% relative (expressed in gradient time)
6	Mobile phase flow rate	ml/min	±0.1 absolute
7	Concentration of the buffer	mM	±10% relative
8	Injection volume	µl	±25% relative
9	Concentration of additives	mM or %	±10% relative
10	Detection wavelength	nm	±3 nm absolute
11	Source of the organic solvent	Vendors	Two different vendors
12	Equipment	Vendors	Two different vendors of the same quality equipment
13	Others, . . .		

should be evaluated case by case. The significance of the effects of the factors on the responses, such as the resolution of all peak pairs, the tailing factor, retention times, analysis time, etc., is evaluated.

C. Sample Preparation Parameters

A tentative list of factors to be evaluated in robustness testing for sample preparation is presented in Table 4. Additional factors can be evaluated and other limits applied. Responses to be considered are usually related to the quantitative aspects of the method. The recovery of the main compound (100%) and all relevant impurities spiked at e.g. 0.5% w/w level in a reference solution prepared according to the conditions described per experimental run of the design, are calculated against a freshly prepared reference solution as described in the method description. This is done to demonstrate effects of the studied parameters on the recovery. The recovery of the main compound (100%) and all relevant impurities spiked at 0.5% w/w level in a recent- and worst-case (old) sample batch, is calculated against a

TABLE 4 Sample Preparation Factors Potentially Investigated in a Robustness Study

	Factor	Units	Limits of factor levels
1	Percentage of organic modifier in the extraction solvent	%	±10% relative
2	Sample matrix concentration	mg or units	±10% relative or ±10 mg absolute or adding yes/no of 1 placebo solid unit
3	pH of the extraction buffer	Units	±0.1 absolute
4	Concentration of the extraction buffer	mM or mg	±10% relative or ±10 mg absolute
5	Mixing and equilibration times	min	±10% relative
6	Filter type	Vendors	Two different vendors
7	Extraction temperature	°C	±5 absolute
8	Dilution ratio	Different volume ratio's with similar final concentration	A minimal volume ratio and a maximal volume ratio (e.g. 1 ml stock/10 ml final volume and 10 ml stock/100 ml final volume)
9	Type of mixing	Mixing procedure	Shaking/sonication[a]
10	Shaking speed	RPM	±10% relative
11	Blending time	min	±10% relative
12	Concentration of active	mg	±10% relative or ±5 mg absolute
13	Filtration pressure	Filtration purge	Gradual/fast
14	Discard volume at filtration	ml	1–6 ml, absolute
15	Equipment used in sample preparation (mechanic shakers, blenders, etc.)	Different vendors	Two world wide available vendors
16	Others, . . .		

[a]Sonication should be avoided in sample preparation procedures of final methods.

freshly prepared reference solution as described in the method description. This is done to demonstrate effects of the studied parameters on the recovery in the presence of sample matrix. Additionally, the recovery of the main compound (100%) and all relevant impurities spiked at 0.5% w/w level of a recent- and worst-case sample batch, may be calculated against a reference solution prepared according to the conditions as described per experimental run of the design. This is done to demonstrate effects of the studied parameters on the extraction of the main compound and the relevant impurities. In addition, depending on the situation, other responses such as RT recovery, etc. may also be considered.

D. System Suitability Test Limits

After data analysis, the statistically significant effects induced by certain factors are identified. This is typically done by using standardized Pareto plots (Figure 2). This plot shows the standard values of the effects in descending order of magnitude. The length of each bar is proportional to the standardized effect, which is equal to the effect divided by the standard error. This is identical to the calculation of a *t*-value for each effect in a Student *t*-test. The vertical line in the plot is used to decide which effects are significant. This line corresponds with the boundary of a 99% reliability level. Each effect that transgresses this vertical line is considered to be significant.

A statistically significant effect is not always relevant from the practical point of view. Therefore, a worst-case level combination experiment with regard to the studied response (e.g. resolution) is determined and performed with replicates. In this experiment, only the method parameters with major effects (both statistically significant and almost significant) are considered. As can be observed in Figure 3, the major effects are easily detected as being the temperature and the pH. The worst-case level combination for resolution would have been conditions at low temperature and pH (decrease in resolution). The response of this worst-case experiment is then compared with the system suitability limit (if available) by a *t*-test to investigate whether the system suitability limit has the potential to be violated. When this occurs, the suspected factors are investigated quantitatively in order to describe their influence in more detail. In such a case, limits of tolerable factor levels are defined clearly for the critical factors so that unexpected problems can be avoided. In the case where the system suitability limits are not yet set, it is possible to use the response of the worst-case experiment to determine appropriate limits that are more practical.

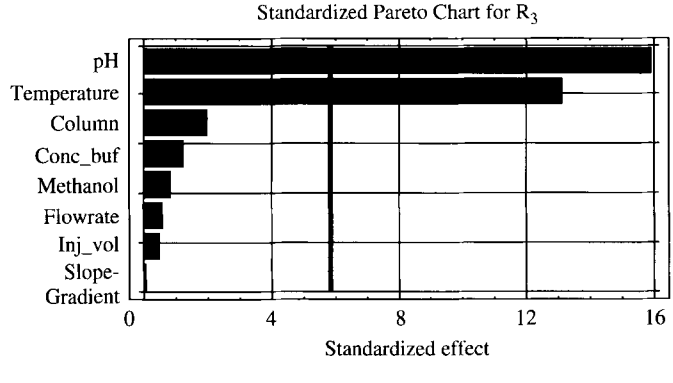

FIGURE 2 Standardized Pareto plot. The standardized effects are plotted in function of their extends. The dark line is the border of decision according to a t-test. Critical effects exceed this line.

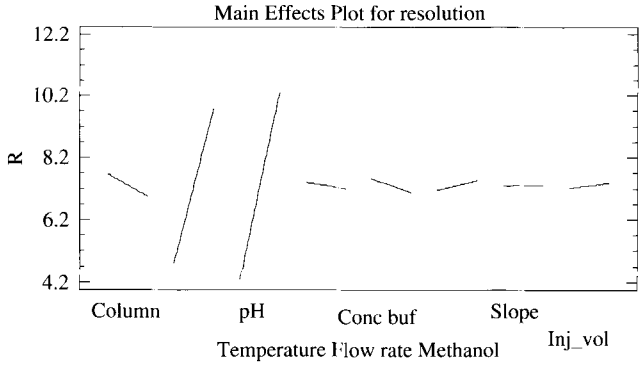

FIGURE 3 An example of a main effect plot. The average responses at low level and high level of the factors are plotted for each experimental factor studied.

VI. CONCLUSIONS

Method validation is expected to be the finishing touch on a proper method development process. During validation, there should be no surprises toward failures on the method performance. Usually method validation is covered by standard procedure approaches that are executed within a GMP compliance environment. The validation is documented in a validation report summarizing the obtained validation results.

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TROUBLESHOOTING IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Troubleshooting HPLC instrumentation and separations require a fundamental understanding of how the instrument functions and how the separation works. This chapter provides a practical guide to common HPLC problems, along with more in-depth information to help the reader understand the relationships between the observed symptoms

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and the underlying causes. The practical approach presented here is meant to serve as both a troubleshooting guide and an HPLC learning tool.

I. INTRODUCTION

This chapter provides an overview of HPLC troubleshooting and summarizes guidelines for system maintenance. Troubleshooting problems are classified into four major categories: (1) problems observed in the chromatogram; (2) operating parameters; (3) leaks; and (4) pressure problems. The chapter concludes with a section on how to avoid problems by routine and regular maintenance. Routine maintenance procedures that can be performed by the user are emphasized. After each problem is described, symptoms and possible solutions are proposed. The reader is also referred to textbooks,[1–4,7] a magazine column,[5] and a computer training program.[6] We will discuss primarily reversed-phase (RP) column systems since they are predominantly used today in the pharmaceutical industry. Rather than reading this chapter in a linear fashion, the reader is encouraged to find the relevant problem area in the outline, go to that section, and read about possible causes. Solutions to the problem (if not obvious) are added in italics.

II. PROBLEMS OBSERVED IN CHROMATOGRAMS

Other than obvious problems (leaks, no flow, no signal), the chromatogram is the most common indicator of problems requiring attention. Poor resolution, broad peaks, too few or too many peaks are typical problems observed on the chromatogram (Figure 1).

With current data systems, a variety of chromatographic performance characteristics can easily be recorded and printed out. These include

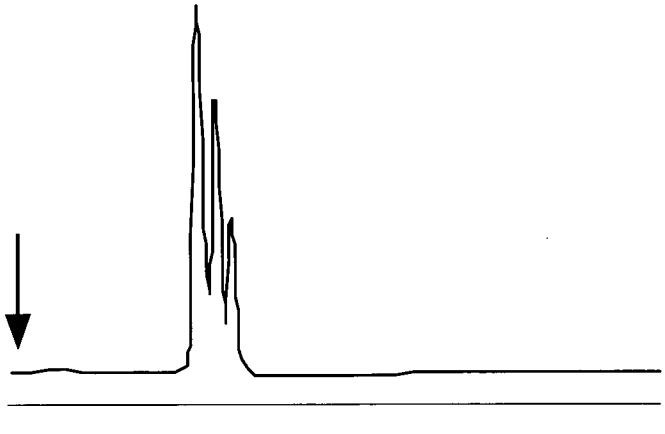

FIGURE 1 Poor resolution.

retention times, peak areas, peak heights, asymmetry factor, plate counts, selectivity values, and resolution. It is useful to record all of these data when developing methods, and then record again on a regular basis (either weekly or monthly). Major changes in any of these parameters may (probably will) require action on your part.

A. Peak Problems

No peaks usually indicate an instrumental problem (Figure 2). "No peaks" could be due to a number of causes including no sample injected, system not turned on properly, major leaks, a dead detector, wrong mobile phase, or a particularly retentive or adsorptive column. Check and measure (use a stop watch and graduated cylinder) flow rate. No flow usually indicates pump problems, flow system blockages, or empty solvent reservoirs. Then check for leaks; if any, eliminate them. *Inject an un-retained compound like uracil for reversed-phase systems*. If there is flow and no peak for uracil, there is a major instrumental, system problem. *Keep reading.*

Too Many Peaks: This usually occurs from sample carryover, usually in the autosampler or on the column (Figure 3). Using a sample solvent stronger than the mobile phase may cause sample precipitation in the column. The next blank run will show the original chromatogram, but usually at a lower concentration. Clean the column! Extra peaks could also result from the degradation of an unstable component. Investigate

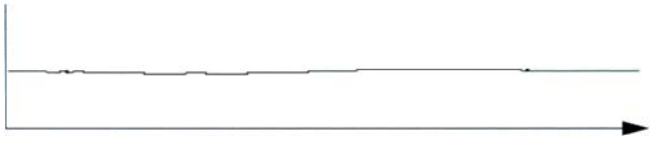

FIGURE 2 No peaks.

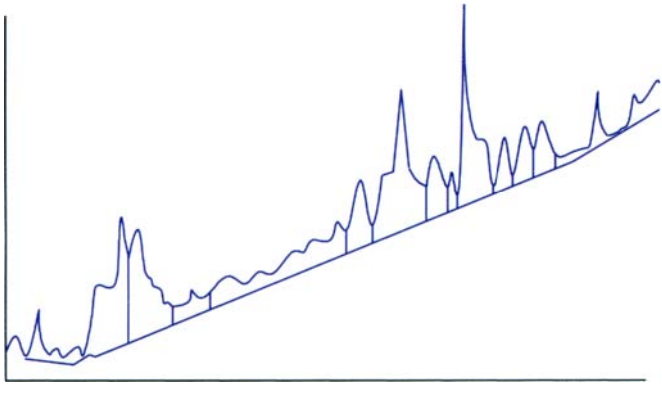

FIGURE 3 Too many (extra) peaks.

the stability of suspected peaks with respect to time, pH, and light. After the first injection, some sample vial septa may produce an extra peak in subsequent injections from the same vial. This is usually a plastisizer extracted from the septa. *Change the type of septa.*

Isocratic Elution: If the extra peaks are broader than in the original sample, the current conditions (run time, mobile phase) do not elute all the components. Either wait until all peaks elute, use a stronger mobile phase, or use a gradient elution method.

Gradient Elution: Run a blank gradient – if the extra peaks still occur they are probably impurities in the solvent. Make up new mobile phase. *Always buy and use HPLC grade solvents.*

If no peaks occur in the blank, but all peaks in duplicate injections show broad peaks or doublets, this indicates a channel in the column, usually caused by the dissolution of silica by extreme pH conditions. *Install a new column.*

Too Few Peaks: This is rare and most often indicates poor resolution, i.e. one or more peaks are overlapping (unresolved) with other peaks. *Increase the resolution or use "peak purity" software with a PDA to confirm presence of unresolved peaks.* Loss of some peaks or changes in peak area may also indicate a "bad" or active column that irreversibly adsorbs the peak, often a basic peak with silica-based columns.

Fronting and Tailing Peaks: Fronting peaks are usually caused by column overload (sample amount exceeding the sample capacity of the column); dilute the sample 1/10 and re-inject. Tailing peaks are caused by secondary (undesirable) interactions of the analyte with the stationary phase, or a poor connection (excess void volume). Check to make sure all fittings and tubing between the injector and detector are correct. A common example of acid/base interaction is the tailing of basic analytes caused by strong interaction with the acidic residual silanol groups of silica-based bonded phases. This can be reduced by adding an amine modifier (20 mM triethylamine or TEA) to the mobile phase, or by replacing the column with a base deactivated or more inert column. Be sure to adjust and record the pH of the mobile phase after adding the TEA. Asymmetric peak shapes can also be caused by degradation of the analyte during chromatography (Figure 4).

Negative and Positive Peaks: Positive and negative peaks (dips) in the chromatogram are common with an refractive index (R.I.) detector. Negative peaks at the solvent front are caused by refractive index changes with UV detectors or by sample solvents with less absorbance than the mobile phase. These baseline perturbations are usually ignored by starting data integration after the solvent front. If all peaks are negative, it might be an indication of the wrong polarity in the detector output signal. Additionally, the choice of reference wavelength should be examined. If the analyte absorption is greater at the reference wavelength than at the monitored wavelength, negative peaks will be observed.

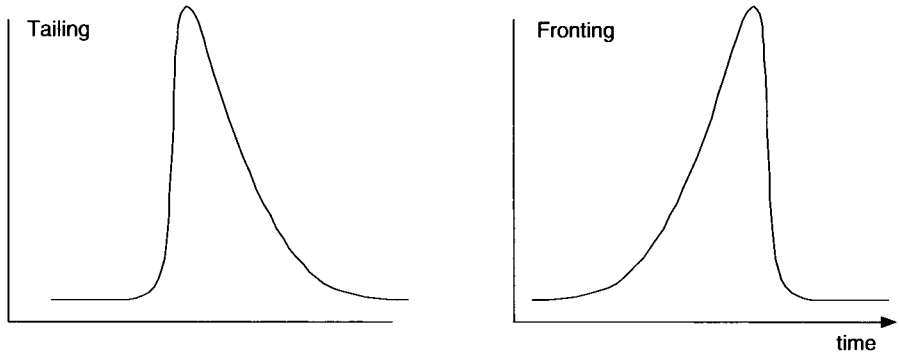

FIGURE 4 Different peak shapes.

Broad Peaks and Split Peaks: Broad peaks and split peaks are indications of degraded column performance caused by sample contamination, a partially blocked inlet frit, or column voids caused by dissolution of silica particles, usually at high pH. *Replace the column.* Another alternative to column replacement is to attempt to clean the blocked frit. Reverse the direction of flow through the column for 2 min. Allow the column effluent (now coming out of the "inlet" of the column) to go directly to waste *and not through your detector*! This will remedy many peak splitting problems. Note that not all columns can be reversed. Some columns have different size frits on the inlet and outlet of the column. Anomalous peak shapes can also be caused by injecting samples dissolved in solvents stronger than the mobile phase. If possible, the sample solvent should be of weaker strength than the mobile phase or of equal strength to the mobile phase. If strong solvents must be used, the volume should be kept small (e.g. <5 μL) to prevent peak broadening or peak splitting of the early eluting peaks.

B. Changes in Retention Time

Changes in flow rate and mobile phase composition are the major factors responsible for changes in retention time (t_R) (Figure 5). If the mobile phase composition changes, the retention times of all peaks will change *except* for the t_0. The t_0 is only affected by the flow rate. Therefore, if your t_0 changes, your flow rate has changed. This is most likely a pump problem (check valve or pump seal problems). If t_0 is constant and the rest of the peaks move, the mobile phase composition has changed. Check that the correct mobile phase composition is being fed to the column. If you suspect the wrong pH or wrong mobile phase composition, do not waste time trying to verify this, *just make up a new mobile phase.* Always prepare and measure pH in the aqueous portion of the mobile phase before adding the organic solvent. Organic solvents change the pH. Table 1 lists the most common buffers used to adjust pH.

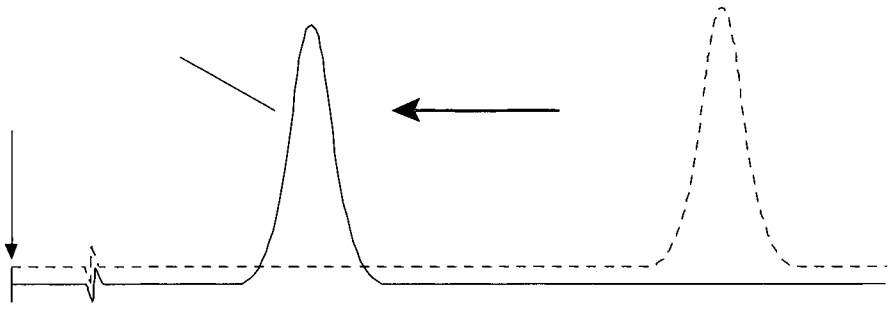

FIGURE 5 Changes in retention time.

TABLE 1 Recommended HPLC Buffers

pH value	Buffer/modifier	UV cut-off (nm)[a]
2.0–3.0	Phosphate	210
2.5–7.5	Citrate	250
3.5–6.0	Acetate	230–240
6.0–8.5	Phosphate	210
7.0–9.5	"TRIS" (tris-hydroxymethylaminomethane)	220–225
8.0–10.5	Borate	210
9.0–12.0	Diethylamine (fresh!)	

[a]These values apply to about 50 mM of buffer.

Wrong pH: The buffer could be too weak; *use a 20–100 mM buffer when possible.* Never chromatograph weak acids or weak bases with a pH close to their pK_a (they will be only partially ionized and may show tailing or badly shaped peaks in addition to irreproducible retention times). *Use a pH ± 2 units above or below analyte pK_a for good reproducibility.*

Wrong Mobile Phase: Too much organic (methanol or acetonitrile) in reversed-phase separations will cause a decrease in all retention times. *Make new mobile phase.*

In gradient elution, lack of equilibration between runs often produces shorter retention times only for early peaks. Confirm equilibrium by measuring retention time stability by making three or four replicate runs. High organic or aqueous content (>95%) causes some phase collapse and may require longer equilibration times between gradient runs. *Wait longer between runs or start with a stronger mobile phase initially.*

Wrong Flow Rate: There is an optimal flow rate for each column, typically about 1.0 mL/min for a 4.6 mm i.d. column. Retention time is inversely proportional to flow rate, *so measure, and adjust if necessary, the flow rate.* An easy way to shorten the analysis times for most methods is to increase the flow rate to 1.5 or 2.0 mL/min if the pressure allows.

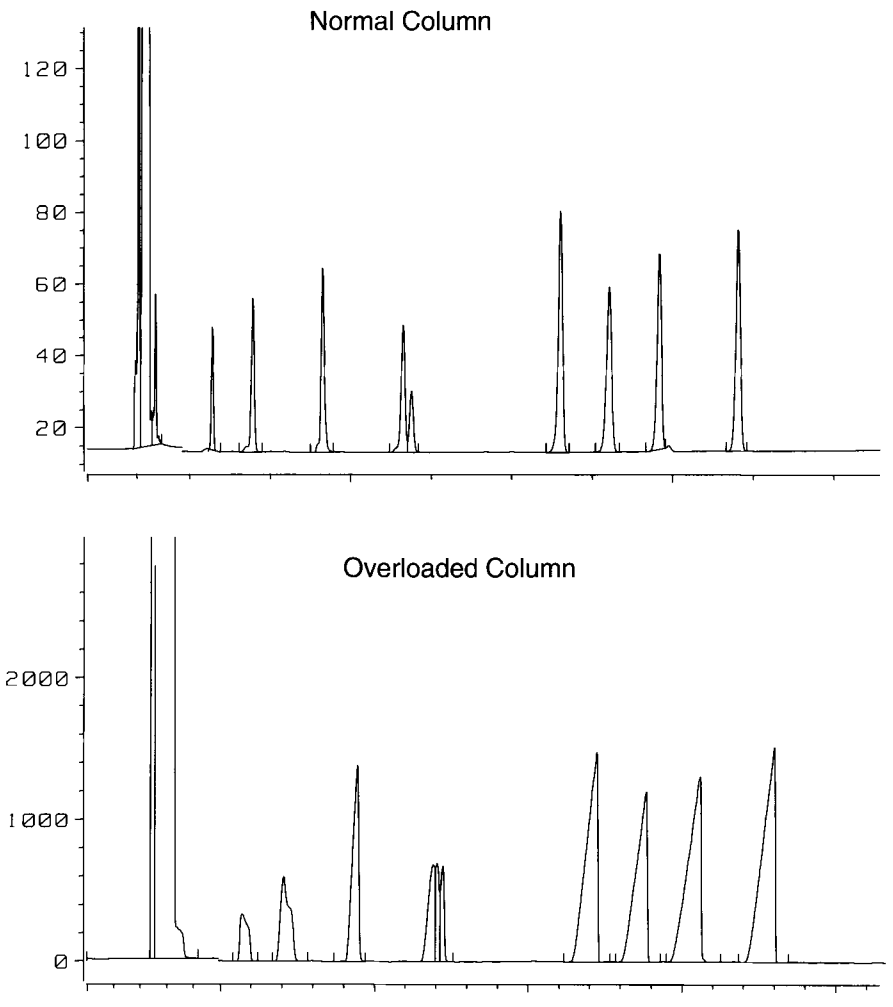

FIGURE 6 **Overloaded column.**

Decreasing retention time with fronting peaks often indicates an overloaded column (Figure 6). Dilute the sample 1/10 and re-inject.

Retention time stability only after a few initial injections indicates active sites in the column. One possibility is that a polar molecule in the sample temporarily covers (adsorbs onto) these sites. *Purchase a more inert column, or add a mobile phase modifier like TEA to cover up active silanol sites.*

C. Peak Area Problems

Reproducible peak areas are essential for good LC performance. Expected precision is a function of analyte activity, sample concentration,

TABLE 2 Typical Retention Time and Peak Area Precision

Peak retention time precision	
With oven	≤0.3%
Without oven	≤0.7%
Peak area precision	≤1.5%

TABLE 3 Column Efficiency (*N*)*

Length (cm)	Particle diameter (μm)	Efficiency (*N*)
25	10	10,000
25	5	22,000
15	5	14,000
10	5	10,000
10	3	12,000

*Experimental data – H. McNair's Laboratory.

detector, flow rate stability, and data system. Usually one can expect a %RSD of 1% or less for six replicate injections of a standard of reasonable concentration down to about 10 ppm (w/v). Lower concentrations often exhibit higher RSDs due to problems in precision of injection techniques and/or LODs of the detector and data system (Table 2).

Peak area changes may also be due to changes in volume injected, flow rate, wavelength of detector, pH, leaks, sample stability, integration problems, and partial loss of sample due to irreversible adsorption on a dirty frit or active column.

Time spent in method development, in choosing a proper column, pH, mobile phase, even column temperature, to produce symmetrical peaks usually results in better precision of peak areas.

If only one peak in a mixture shows poor precision, possible causes could be wrong pH (analyte is only partially ionized), large peak asymmetry causing integration errors (try a more inert column, or a mobile phase modifier). If retention factor (k) is too low (<2), the peak may be overlapping with other peaks. *Decrease the strength of mobile phase.*

D. Broad Asymmetrical Peaks

If all peaks are broad, the column efficiency (N) may be too low. *Try a longer column or smaller particle diameter or use the optimal flow rate.* Table 3 discusses plate number N as a function of column length and particle diameter.

E. Noisy Baseline

1. Short-Term Noise

Short-term noise, if detector related, is very small. Modern UV detectors have noise specifications of about $\pm 1 \times 10^{-5}$ AU. More noisy signals, like of Figure 7a, often originate with the low energy of an aging UV lamp. UV lamps should be replaced regularly, after a specified number of operating hours, or after a certain time period. Some short-term noise is normal at high sensitivity.

2. Synchronous Noise

Synchronous noise (Figure 7b) is a periodic noise usually associated with pump strokes. With a piston volume of 100 µL, a flow rate of 1.0 mL/min will generate noise spikes every 6 s. In order to verify that the noise is caused by the pump, turn off the pump. If these periodic noises disappear, they came from the pump. Pump malfunctions, leaking check valves, leaking pump seals, a faulty pulse dampener, or air bubbles are

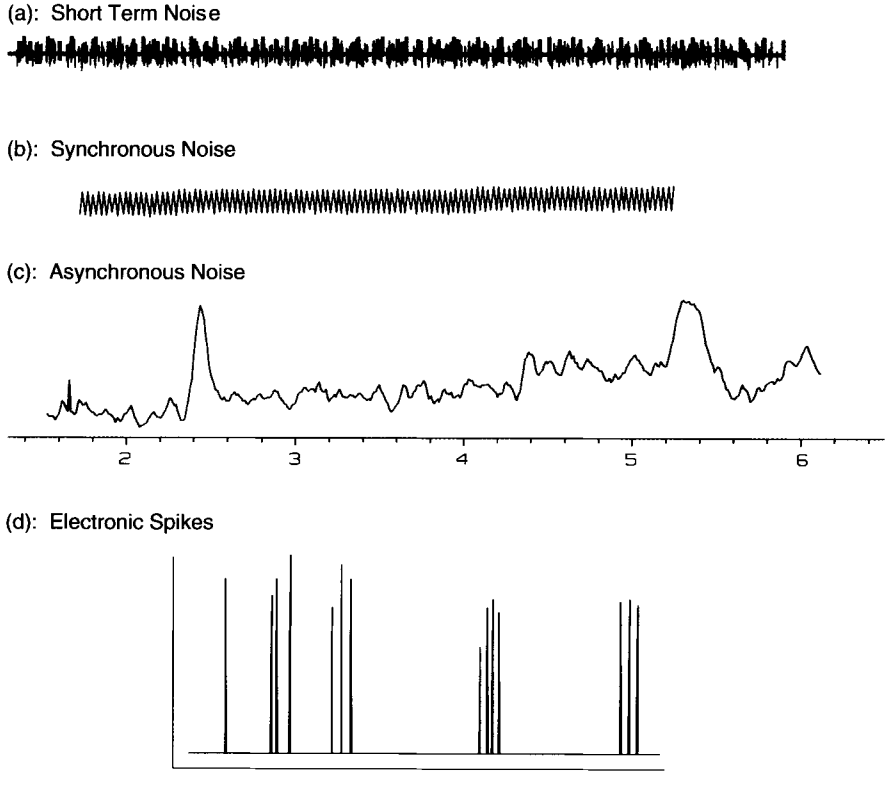

FIGURE 7 Typical noise problems: (a) short-term noise, (b) synchronous noise, (c) asynchronous noise, and (d) electronic spikes.

also possible causes. Air bubbles passing through the pump are the most frequent cause of synchronous or periodic noise. Modern LC systems use in-line vacuum degassing. This is the best way to avoid air bubbles. Helium sparging is not as effective, and requires a helium cylinder. Helium does prevent bacterial growth in buffers.

3. Asynchronous Noise

Asynchronous noise (Figure 7c) is random noise often associated with contamination of mobile phases, a dirty or leaky detector cell, poor mixing of gradients, sample degradation, leaks or late eluting impurities in samples or solvents. Once again, turn off the pump to verify that it is not a pump problem.

4. Very Fast Synchronous or Non-Synchronous Noise Spikes

Very fast synchronous or non-synchronous noise spikes (see Figure 7d) are caused by electrical problems such as power/voltage fluctuations, poor grounding, or loose detector/data system connections. If not obvious, the best remedy is to call a service engineer (see Section VI for routine items to check before calling the service engineer). These spikes could also be due to air bubbles in the detector cell, less likely with the new generation of in-line vacuum degassing systems.

5. Baseline Drift

Baseline drift often reflects lack of temperature equilibrium, or changes in the energy output of the UV lamp. This baseline drift is reduced significantly in dual-beam or reference wavelength absorbance systems. Baseline shifts associated with gradients are normal, indicating more absorption in the stronger solvent (particularly when methanol is used as the strong solvent). Non-specific drifts, up and down, can be caused by strongly retained peaks slowly eluting off a contaminated column.

III. OPERATING PARAMETERS

A. pH

With the exception of normal phase and GPC, all other forms of HPLC include water in the mobile phase. In almost all cases, the pH should be carefully controlled. If no buffer is used, the pH of the water component can easily drift due to absorption of CO_2 from the atmosphere or due to extraction of impurities from sample loops, column frits, and dirty columns.

Ammonium phosphate is a very popular buffer for several reasons: (1) its pH range of 2.0–3.0 neutralizes all weak acids, and fully ionizes all weak bases; thus it is versatile; (2) it is readily available in high purity; (3) it has a high solubility with high concentrations of acetonitrile, i.e. no

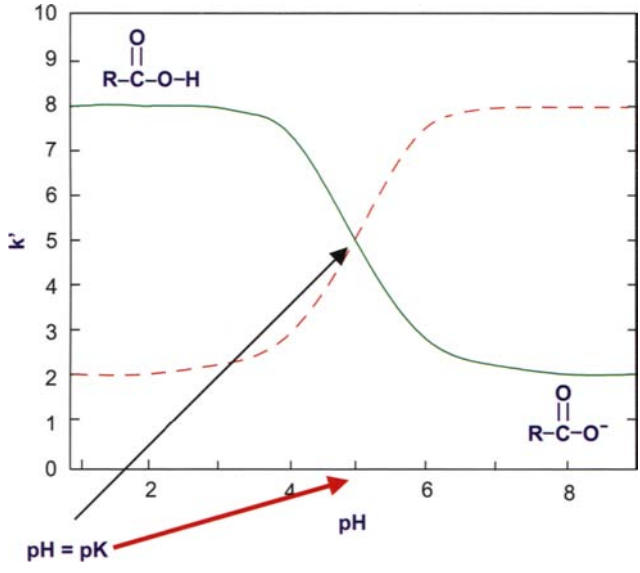

FIGURE 8 **Dissociation curves for a weak acid and a weak base.**

precipitation; and (4) its UV cut-off is about 210 mm. Phosphate buffers should not be used, however, when interfacing to an LC/MS system because they will contaminate the ion source. For LC/MS, use the more volatile acetate buffers (Figure 8).

As mentioned earlier, chromatography with pH close to the pK_a of any analyte is to be avoided. Use a pH $\pm$ 2 units removed from the pK_a.

Addition of organic mobile phase changes the pH of aqueous solvents. It is recommended to measure pH before adding organic solvents. Remember buffers almost always increase UV absorption, which could be a problem in gradient elution. Buffers are an enemy of pump seals (if precipitation occurs), and buffers shorten the lifetime of silica-based columns.

In theory, pH affects only weakly ionized acids and bases; neutral molecules and strong acids and bases are not affected. However, pH can affect the stationary phase if it is silica based. To minimize the effect of high pH on silica gel-based columns: (1) work at room temperature; (2) use a densely bonded, highly end-capped reversed-phase column; and (3) use a high percentage of organic component in the mobile phase as this lowers the "effective" pH.

The silica surface has silanol groups, which are polar and weakly acidic (Figure 9). In normal phase (pure silica), these silanols are responsible for the necessary polar interactions resulting in separations. In bonded-phase packings, both normal and reversed phase, columns still contain unreacted

The surface of silica gel is *nonhomogeneous*, being comprised of several different types of functional groups:

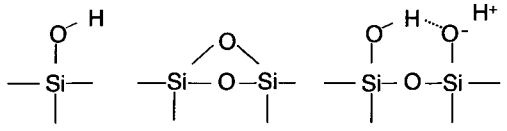

FIGURE 9 Silanols on silica surface.

silanols groups. Less than 50% of all silanols can be reacted with organochlorosilanes to form bonded phases. Unless thoroughly end-capped or one of the newer "hybrid" columns are used, these silanols often cause problems with basic compounds. To check for silanol activity, inject pyridine and examine the peak shape. Only a well-deactivated column will produce a symmetrical peak. It may be necessary to use a low pH, 2–3, in order to neutralize any free silanol group.

B. Mobile Phases

Use only HPLC grade solvents. This is the only standard acceptable for the exacting requirements of modern HPLC systems. Record the lot number in case a problem of quality does arise.

If you use an in-house HPLC grade water system, compare the quality of that water with a commercial grade HPLC water. Pump 100 mL of your in-house water through an RP-18 column (this is your sample); all of the organic impurities are adsorbed on the column. Now program the methanol concentration from 0% to 90% in 9 min and record the peak area with a 254 nm UV detector. There will be a large ugly peak due to the large amount of organic contaminants in 100 mL of water. Compare this peak area to that of the commercial grade water. Use the water that has the fewest impurities.

C. Stationary Phases

Here is a Figure 10 of the most common phases.

D. Particle Diameter (d_p)

The particle diameter affects many chromatographic problems but is not a source of problems in itself. The quality of current day LC packings is outstanding. Smaller particles provide more plates (N) and result in better resolution (refer to Table 3). However, the pump pressure required

	REVERSED-PHASE (AND ION-PAIR) METHOD
C-18 (octadecyl or ODS)	Rugged; highly retentive; widely available
C-8 (octyl-)	Similar to, but slightly less retentive, than C-18
C-3, C-4	Less retentive; used mostly for peptides and proteins
C-1 (trimethyl-silyl, TMS)	Least retentive; least stable
Phenyl	Moderately retentive; some selectivity differences
CN (cyano)	Moderately retentive; used for both reversed- and normal-phase
NH_2-(amino)	Weak retention; used for carbohydrates; less stable
Polystyrene	Stable with 1 < pH < 13 mobile phases; better peak shape and longer column life for some separations
	NORMAL-PHASE METHOD
CCN- (cyano)	Rugged; fairly polar; general utility
OH- (diol)	More polar than CN-
NH_2- (amino)	Highly polar; less stable
Silica	Very rugged; cheap; less convenient to operate; used in prep LC

(a)

	SIZE-EXCLUSION METHOD
Silica	Very rugged; adsorptive
Silanized silica	Less adsorptive, wide solvent compatibility; with organic solvents
OH- (diol)	Less stable; used in aqueous SEC (gel filtration)
Polystyrene	Used widely for organic SEC (GPC); incompatible with highly polar solvents
	ION-EXCHANGE METHOD
Bonded-phase	Less stable and reproducible
Polystyrene	Less efficient; stable; more reproducible

(b)

FIGURE 10 (a) Common column packings and (b) common column packings. (Reprinted from L.S. Snyder, J.L. Glajch and J.L. Kirkland, Practical HPLC Method Development, J. Wiley (1988) 80–105 with permission.)

to maintain flow rate is inversely proportional to the particle diameter squared, *thus* you will rarely see a long column packed (>15 cm) with small particles (<3.5 μm) unless you have a high-pressure pump (Figure 11).

E. Column Length

Both plate number N and pressure drop are proportional to column length. Twenty years ago most columns were 25 cm in length packed with 10 μm particles. As high-quality smaller particles (no easy task to

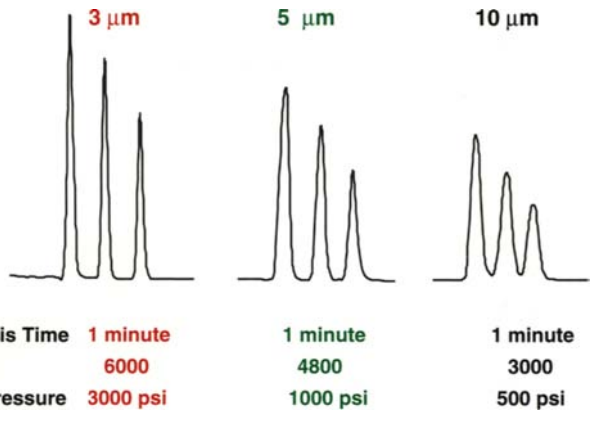

FIGURE 11 Particle diameter and backpressure.

manufacture) became available, column lengths became shorter. Since in isocratic mode analysis time is proportional to column length, the current trend is for faster analyses using shorter columns packed with smaller particles (Figure 12).

F. Flow Rate

Van Deemter type plots (discussed earlier in Chapter 2) show the effect of linear velocity on band broadening (HETP). Each column has an optimal flow rate to produce a minimum in band broadening. Figure 13 shows a typical such plot for 10, 5, and 3 μm particles. Note that this is a fairly minor effect with small particles (1.8, 3.0, 3.5, and even 5 μm particles) (Figure 13).

Optimal flow rates are approximately 0.8, 1.2, and 2.5 mL/min for the 10, 5, and 3 μm columns (assuming an internal diameter of 4.6 mm). Thus, smaller particles show faster optimal flow rates. The interesting part, however, is the slope of each plot after the minimum. Smaller particles allow faster than optimal flows with minimal loss in efficiency.

IV. LEAKS

Column leaks are a routine, almost normal occurrence in HPLC; fortunately, they are easily resolved. Since columns are being replaced on a regular basis, column leaks, usually at the inlet side (higher pressures), are the most common problem. We recommend using PEEK ferrules and universal (finger tight) nuts (Figure 14).

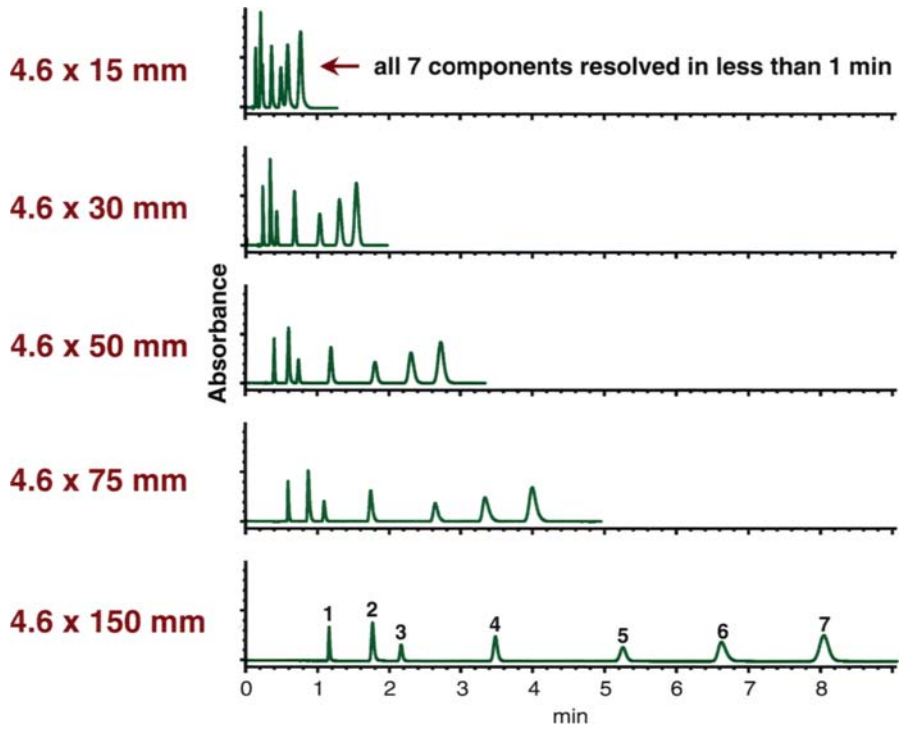

FIGURE 12 Effect of column length on retention time.

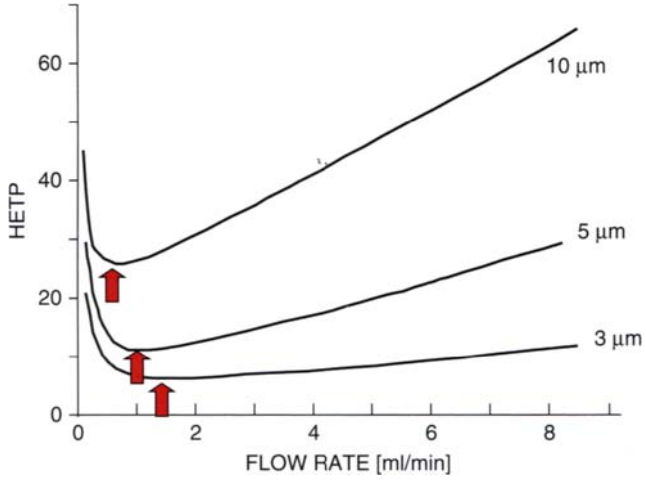

FIGURE 13 Van Deemter plots 10, 5, and 3 µm.

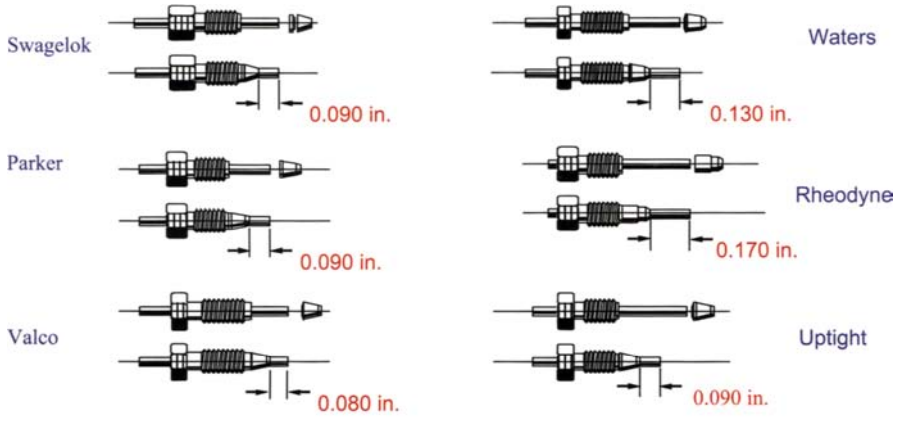

FIGURE 14 HPLC fittings. (Reprinted from J.W. Dolan and P. Upchurch, LC/GC Magazine 6:788 (1988) with permission.)

First make sure the column fittings are tight. If they are tight and leaks still occur, loosen the fitting and start again. If you still have leaks, replace that fitting and the associated hardware. Replace stripped or dirty fittings with new fittings.

No visible leak but white powder (residue of buffers) at any system connection indicates a leak. First, tighten, then, if necessary, cut the tubing and replace all hardware.

Pump leaks arise from loose check valves (tighten, if necessary replace), loose inlet or outlet fittings, pump seal failure, pressure transducer failure, or purge valve failure. With a proper manual and minimal training, most workers can replace check valves, filter, pump seals, detector lamps, flow cells, autosampler syringes, and manual injector rotor seals. Most pharmaceutical laboratories will have service contracts that provide both emergency and routine maintenance service.

Injector leaks usually occur because of loose connections, dirty or plugged sample loops, rotor seal failure, an improper or broken syringe, wrong syringe needle type, and/or blocked or elevated waste line.

Detector leaks arise from cell gasket failure, loose connections, blocked outlet line, or a blocked or dirty flow cell.

V. PRESSURE PROBLEMS

Too high pressure is most often caused by partial blockages (listed in order) in filters, column inlet frits, sample loop, column outlet frit, or detector. To isolate the problem, disconnect all components coming after

the pump in the flow system. Add one at a time, in the proper order: connecting tubing, sample valve (or autosampler), guard column, analytical column, detector, any post-detector device (second detector, split valve, or sample collection device). Repair or replace the component responsible for the high pressure.

Columns with small particles (3 μm and smaller) generate higher than normal pumping pressures; that is why they are usually short in length, 5 or 10 cm.

Smaller particle columns also develop blockages earlier than larger particles, so smaller inline filters (0.5 mm i.d.) are commonly used. If the high-pressure blockage occurs in the column, the most likely culprit is the column inlet frit. Clean or replace this frit if possible. If the column itself is the problem, disconnect the column from the detector, reverse the direction of flow (most blockages are in the column inlet side), and flush with 50/50 water and methanol at 60°C. This elevated temperature is used to more easily dissolve precipitates of buffers and samples. After 10–20 column volumes increase to stronger solvents, i.e. 90% methanol, then 90% tetrahydrofuran (THF).

Low, or no pressure, is caused by leaks (piston seal, column connections, injector), pump malfunctions (lost prime, air bubbles in pump head, vapor lock, faulty check valves, broken piston), or inadequate solvent supply (empty solvent reservoir, plugged solvent sinker, crimped solvent lines, or wrong solvent mixture).

Pressure cycling is caused by air bubbles in check valves (remedied by degassing the solvent), malfunctioning of check valves or pulse dampeners, or partial system blockages (Table 4).

VI. TROUBLESHOOTING ACTION PLAN

Before calling the service representative check the following:

- Instrument on?
- Instruments(s) plugged in?
- Fuse(s) blown?
- Other equipment on the same electrical circuit?
- No mobile phase?
- Air in the mobile phase reservoir pump line?
- Gas bubbles in pump head(s)?
- Broken pump plunger?
- Worn or leaking pump seal?
- No sample being introduced?
- Solvent incompatible with vials?
- Leaking vial caps?
- Leaking sample valve (e.g. crossport scratches on rotor)?
- Injection valve improperly positioned?

- Contaminated or plugged column?
- Wrong column?
- Wrong or no detector reference?
- Dirty detector cell?
- Faulty or failing detector lamp?
- Wrong detector attenuation?
- Broken or cracked detector cell window(s)?
- System leak(s)?
- Review sample's chemistry?
- Read instrument or equipment manual, including troubleshooting guide?

TABLE 4 Summary of Major LC Problems

	Symptom	Probable causes
Chromatography	No peaks	No flow, poor injection, system down, bad data cables
	Too many peaks	Carryover (autosampler), too weak mobile phase (need gradient method)
	Fronting peaks	Column overloaded
	Tailing peaks	Dirty frit or column, bad injection technique, wrong pH
	Change t_R	Flow rate, mobile phase composition
	Noisy baseline	Pump problems, dirty column, old lamp
Leaks	Column leaks	Loose, dirty, or wrong fittings
	Pump leaks	Loose fittings, pump, Mixes, transducer seals
	Injection leaks	Rotor seal failures
		Dirty or blocked loop
		Wrong syringe or needle
Pressure	High pressure	Blockage of frit or column or connections
		Too small particles
		Too high flow rate
		Wrong mobile phase
	Too low pressure	Leaks, low flow rate, channel in column, pump problems
	Pressure cycling	Check valves, pump seal, air bubbles
Poor resolution	N value low	Old column, dirty column, dirty matrix, sample size too large, bad fittings or tubing
	α value low	Wrong stationary phase, wrong mobile phase
	k value too low	Too strong mobile phase, column temperature too high

VII. CONCLUSIONS

HPLC troubleshooting begins with an understanding of the hardware mechanics and the separation fundamentals. This chapter provides a practical approach to hardware troubleshooting (pumps, autosamplers, columns, and detectors). It also provides an introduction to the concept of resolution in HPLC. Resolution (R) between any two peaks requires three things: the proper retention factor (k – a function of the mobile phase strength), the correct selectivity (α – the chemical interaction provided by the stationary phase and the mobile phase), and enough efficiency (N – a mechanical function of the column and packing dimensions). For a more detailed understanding of resolution, please refer to the liquid chromatography chapter of reference (8).

Combining the instrument and separation troubleshooting with the proper preventative maintenance will lead to years of reliable chromatographic data.

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INTERNET RESOURCES

1. https://www.mn-net.com/web/MN-WEB-mnweb.nsf/WebE/CWIK-4P5F9K
2. http://www.metachem.com/tech/Troubleshoot/troubleshoot.htm
3. http://kerouac.pharm.uky.edu/asrg/hplc/troubleshooting.html
4. http://www.waters.com?WatersDivision/pdfs/WA20769.pdf<http://www.waters.com/?WatersDivision/pdfs/WA20769.pdf>
5. http://www.chromatography.co.uk/Techniqs/hplc/trouble1.html
6. http://www.sigmaaldrich.com/Graphics/Supelco/objects/4500/4497.pdf
7. http://www.dq.fct.unl.pt/QOF/hplcts1.html
8. http://www.forumsci.co.il/HPLC/topics.html#Trouble
9. http://www.rheodyne.com/support/product/troubleshooting/index.asp
10. http://www.ChromatographyTraining.com

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18

MOLECULARLY IMPRINTED POLYMERS AS SORBENTS FOR SEPARATIONS AND EXTRACTIONS

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ABSTRACT

Molecularly imprinted polymers (MIPs) are materials that mimic biological receptors with synthetic recognition sites that exhibit pre-determined selectivity toward analyte(s) of interest. MIPs have become increasingly popular in recent years, and have been applied as selective sorbents for extractions and chromatography and in other areas where high specificity is required. This chapter presents a summary of factors of importance in the synthesis and use of MIPs, describes various different synthesis approaches, and reviews recent advances in the field of molecularly imprinted materials.

I. INTRODUCTION

The general concept of molecular imprinting was introduced in 1894 when Emil Fischer presented the idea that the way a substrate interacts

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S. Ahuja and H. Rasmussen, editors.

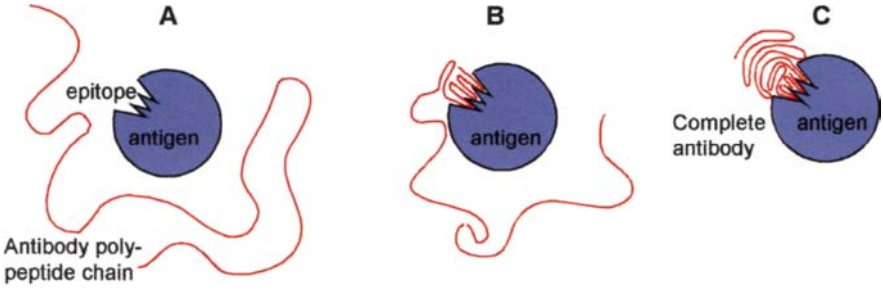


FIGURE 1 **Antibody formation model first proposed by Linus Pauling, which inspired the concept of molecular imprinting. Reprinted from reference 113 with permission.**

with an enzyme is via a "lock-and-key" mechanism,[1] in which an enzyme's active site has a configuration that is complementary to the shape of the substrate. In this analogy, the key (substrate) fits into the lock of enzyme's active site. In a very simplistic view, molecular imprinting can be described as a way of making artificial "locks" for "molecular keys".[2] Just like there is only one key that fits into a particular lock, in molecular imprinting the final polymer has molecular cavities that are specific in shape and orientation only to the template molecule. These "molecular keys" can range from small molecules, such as drug substances, amino acids, steroid hormones, or metal ions, to large molecules, such as peptides or proteins.

In 1940, Linus Pauling introduced his theory on antibody formation,[3] in which the antigen was used as a template in the rearrangement of antibody polypeptide chains. Figure 1 shows the formation of antibody according to Pauling. It is based on the concept that the specificity of an antibody to a particular antigen is dependent upon structural complementarity. Pauling's theory inspired the idea of creating a three-dimensional structure of the antibody polypeptide chain around an antigen template molecule. The synthetic analogs of antibodies were prepared based on the interaction points between antibody and antigen, which can selectively recognize the target molecule. Experiments conducted by Dickey in 1949 are considered the first experimental attempt at molecular imprinting by creating an affinity for dye molecules on silica gel.[4] Imprinting on synthetic organic polymers, however, was not achieved until the early 1970s when covalent imprinting in vinyl polymers was reported.[5–9]

The search for better and more efficient means to prepare, purify, and analyze structurally complex compounds exhibiting chirality, especially in the agricultural chemicals and pharmaceutical industries, has led to the development of molecular imprinting. The concept of MIPs has become increasingly popular in recent years and has entered many areas of chemistry, biochemistry, and biotechnology. Some of MIPs applications include bio- and chemosensor, microreactor, solid-phase extraction (SPE), affinity chromatography, and catalysis.[10]

II. MIP THEORY

Imprinted polymers or MIPs have become increasingly popular and have been applied as artificial antibodies, catalysts, sensors, drug assay tools, and chromatographic stationary phases.[11–16] MIPs are highly cross-linked polymers that deliver spatial and functional memory of the template molecules used to produce them. MIPs are capable of providing a high degree of selectivity when used as sorbents in analytical extractions. A more detailed overview of molecular imprinting can be found in a number of literature reviews on the subject.[17–19a]

MIPs have been shown to possess unique and pre-determined selectivity for target analytes. MIPs can selectively recognize a template molecule used in the imprinting process even in the presence of compounds having similar structure and functionality to the template. Imprinted polymers have been applied for chiral chromatographic separations. For example, an MIP imprinted with L-phenylalanine anilide (L-PA), with methacrylic acid (MAA) as the monomer and ethylene dimethacrylate (EDMA) as the cross-linker was used for phenylalanine separations. The ground and sieved polymers were packed into a 10 cm × 5 mm i.d. column for chromatographic evaluation. Effective separations of L-PA and D-PA were accomplished due to the high selectivity of the sorbents.[19b]

MIPs can be prepared by two general approaches: covalent and non-covalent imprinting. Covalent imprinting was pioneered by Günter Wullf and co-workers at the University of Bonn, and more recently in Düsseldorf, Germany.[2] In covalent imprinting, the functional monomer is covalently bonded to the template molecule prior to polymerization. Then the functionalized monomers are co-polymerized with excess cross-linkers in the presence of porogenic solvents. When the polymerization is completed, the covalent bond between the template molecule and the polymer is chemically cleaved in order to extract the template molecule from the polymer matrix. This results in cavities that are complementary to the template molecule in shape, size, and arrangement of functional groups that serve as recognition sites during rebinding of the imprinted analyte via reversible covalent bonds. However, this technique suffers from lack of general applicability due to the difficulty in finding an appropriate monomer to conjugate to the template.

Currently, the most widely applied technique for preparing imprinted polymers is the non-covalent route developed by Mosbach's group,[7] owing to its generality and simplicity. Non-covalent imprinting utilizes weak intermolecular interactions to self-assemble monomers around the template molecule prior to polymerization. Typical non-covalent interactions include metal–ligand complex, ionic, hydrogen bonding, π–π, dipole, and hydrophobic interactions. Since there is no covalent bonding between the template molecule and the monomers, the template molecule can readily be extracted from the polymer matrix to create the cavities

following polymerization. Although this method is relatively simple, the quality of the resulting polymers depends on the weak interaction between the template and the functional monomers. Stable interactions between template and monomers during polymerization will result in polymers with higher-binding-affinity sites. A broad range of species imprinted has been imprinted via the non-covalent technique during the past 10–15 years as shown in Table 1. Figure 2 shows the procedure for preparing a non-covalent MIP.[20a]

With the non-covalent approach, the selectivity of the MIP can be altered by optimizing the type and amount of functional monomers, cross-linking monomers, and solvent. Careful selection of a porogenic solvent is necessary in order to get a polymer with enough porosity to assure good permeability. The degree of cross-linking is also important for achieving acceptable stability of the template–monomer complex during polymerization, which will also affect polymer porosity. The next section in this chapter discusses some of the most common monomers, cross-linkers, and initiators used for the preparation of MIPs. The synthesis of an MIP can be a challenge due to the various numbers of experimental variables involved. These variables include the nature of the molecule being templated, choice of functional monomers, cross-linkers, porogenic solvents, initiator, method of initiation, and the duration of polymerization. The identification and optimization of the main factors that affect the material structure and its molecular recognition properties are crucial in creating imprinted polymers with high binding affinity.

TABLE 1 A Broad Range of Species Imprinted Via the Non-Covalent Technique. Reprinted from Reference 2 with Permission

Substance class	Examples	Reference
Amino acids	Free and derivitized amino acids	[55]
Peptides	Enkephalin, oxytocin	[114,115]
Steroids	Cholesterol, testosterone, estrogen	[116,117]
Carbohydrates	Derivitized sugars, glycosides	[118,119]
Nucleotides	AMP, IMP	[120,121]
Dyes	Safranine O, rhodanil blue	[122]
Pesticides	Atrazine, 2,4-dichlorophenoxyacetic acid	[62,123]
Metal ions	Cu^{2+}, Ca^{2+}	[124,125]
Drugs	Propanolol, morphine, nicotine, penicillin	[65,126–128]
Proteins	Transferrin, RNaseA, myoglobin, IgG, urokinase	[122,129–132]
Micro-organisms	*Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Listeria monocytogenes*	[133,134]
Crystals	Calcite	[135]

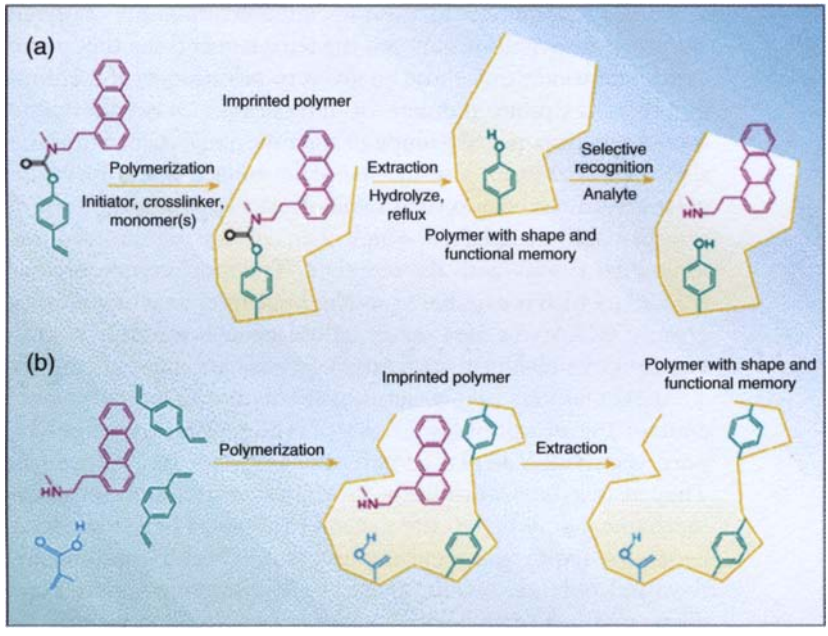


FIGURE 2 **Schematic illustration of (a) covalent and (b) non-covalent imprinting methods. Reprinted from reference 39 with permission.**

The template molecule is the most important factor in the polymerization process. It can be a drug molecule, a specific analyte, or an analog of the target analyte. Template molecules should be chemically inert and stable during free radical polymerization. Critical factors that must be considered before selecting the template molecule are whether the template possesses any polymerizable groups, whether it has any functional groups that can potentially inhibit the free radical process, and whether the template molecule is stable under UV exposure or at higher temperature during the polymerization process. More than one interaction point between template and monomers is required to achieve good binding recognition.[17] The more interactions between the template and the monomers, the better the selectivity. An example of this can be seen in a study of MIP selectivity between phenylalanine derivatives.[20b] As the number of interactions between the template and MAA monomers increases, so does the selectivity exhibited by the MIP. It was also reported earlier that at least three interaction points were required for chiral selectivity. The first two interactions between the enantiomers and the MIP are not specific. Enantiomers were recognized by the MIP based on the third (and higher) interaction. Therefore, the more interactions between the template and monomers, the higher the binding affinity and specificity of the imprinted polymers.

Since recognition in the imprinted binding sites is governed by the attractive interactions between the template and the functional monomers, careful consideration must be given to selection of these monomers.[17] To get the maximum number of interactions between the template and monomers, functional groups of the monomers selected must complement the functional groups of the template molecule. For example, if the template contains a carboxylic or sulfonic acid groups, monomers with amine groups could be selected which then allows for the formation of strong ionic interactions with the template. The most commonly used monomer is MAA, which is capable of strong ionic interactions with basic functional groups. MAA can also serve as hydrogen bond donor and acceptor. In Figure 3, the chemical structures of various monomers are shown.[21,22]

Cross-linkers play a significant role in the imprinting process. These control the morphology of the polymer matrix whether it is a macroporous polymer, a gel type polymer, or a microgel polymer (see Figure 4). They also serve as stabilizers for the imprinted binding sites, and provide mechanical stability in the polymer matrix. High cross-linker ratios are generally required to ensure accessibility and mechanical strength of the final polymer. Some of the most commonly used cross-linkers are illustrated in Figure 5.[17]

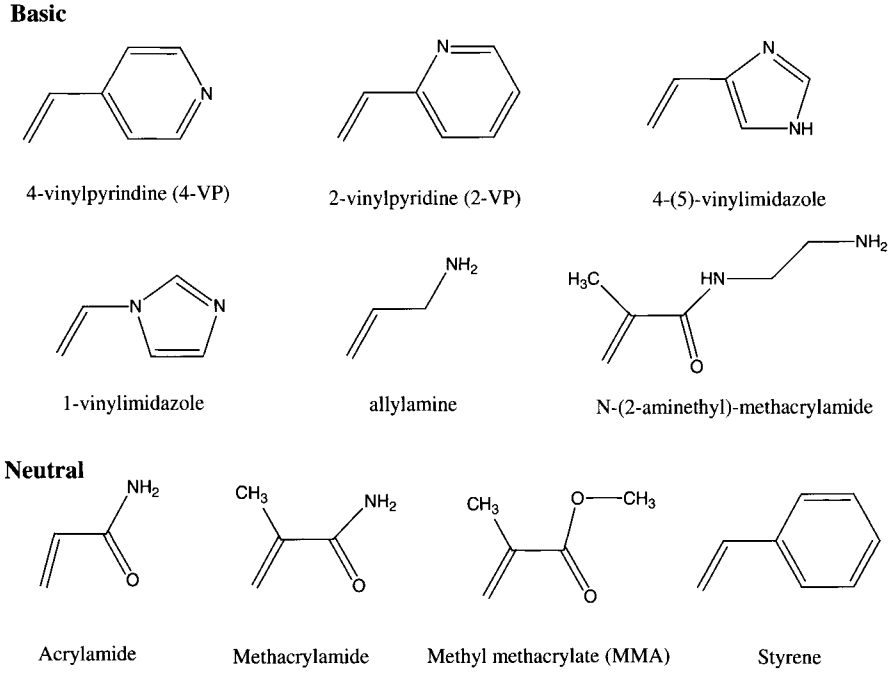


FIGURE 3 A variety of monomers commonly used in the molecular imprinting process.

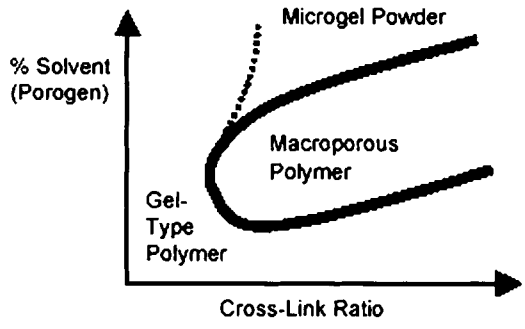
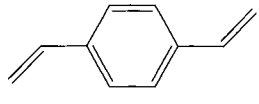
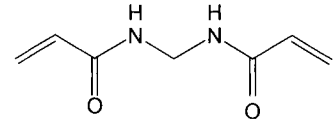


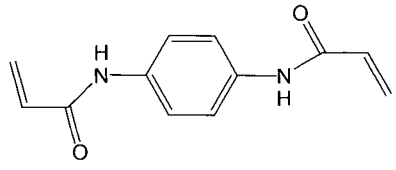
FIGURE 4 **Roles of the porogenic solvent and cross-linker in controlling the morphology of the polymer matrix showed in three distinct regions. Reprinted from reference 17 with permission.**



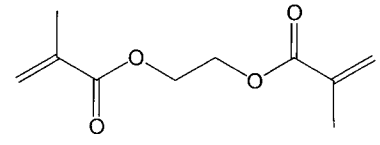
Divinylbenzene



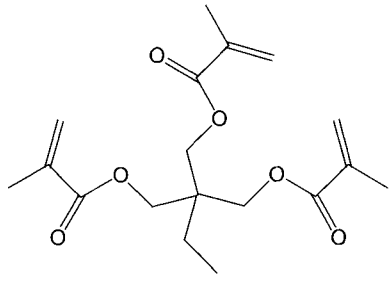
N,N'-Methylene-bisacrylamide



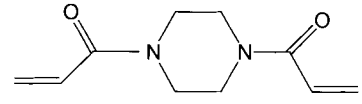
Phenylene-diacrylamide



Ethyleneglycol dimethacrylate



Trimethylolpropane trimethacrylate



Bisacryloyl piperazine

FIGURE 5 **Common cross-linkers used in the molecular imprinting process.**

The porogenic solvent brings all components – the template, monomers, cross-linkers, and initiators – into one phase, while also providing a means for production of pore space within the finished polymer.[17] The choice of solvent is critical to ensure all of the components are dissolved in the pre-polymerization mixture. It is responsible for the success of creating good binding sites and controls the porosity of the final polymer. Aprotic organic solvents with low polarity, e.g. toluene, acetonitrile, chloroform, are generally used to maximize the template–monomers interactions. These solvents are preferred because they can stabilize hydrogen-bonding interactions. However, if hydrophobic interactions are being used, protic solvents such as methanol or water can be selected.

Many chemical initiators can be used as the radical source in the free radical polymerization. Generally, they are used in low levels compared with the monomers. In one study,[23] different concentrations of azobis-isobutyronitrile (AIBN) were introduced into several pre-polymerization mixtures imprinted with 2-aminopyridine to investigate the optimum concentration of AIBN. Results in Figure 6 show that a polymer prepared with 1 mol% AIBN provided the highest MIP binding affinity. The initiation can be triggered in several different ways: thermally, by chemical/electrochemical means, or by photo initiation, depending on the chemical nature of the initiator. For example, AIBN can be initiated by both heat and light (UV) to form radicals that are capable initiating the growth of numbers of vinyl monomers. The chemical structures of selected initiators are shown in Figure 7.

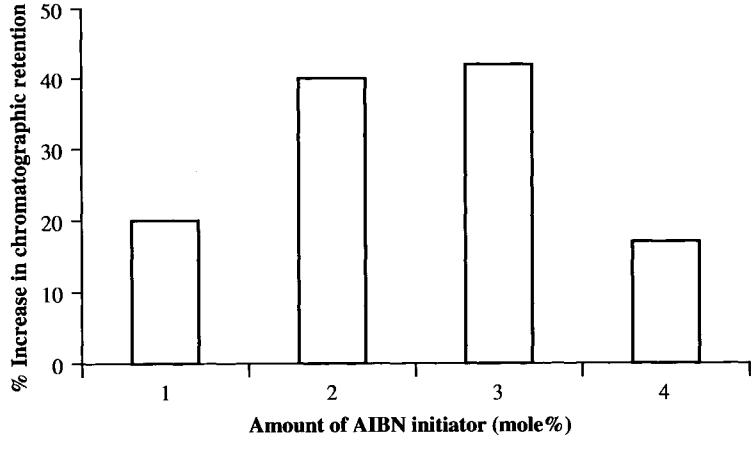
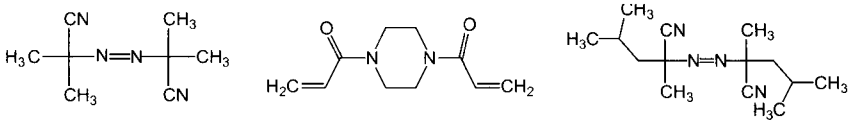


FIGURE 6 **Effect of AIBN amount used in photo-initiation process on MIP binding affinity.**



Azobisisobutyronitrile (AIBN) 1,4-bis(acryloyl)piperazine Azobisdimethylvaleronitrile (ABDV)

FIGURE 7 Chemical structures of selected initiators.

III. SYNTHESIS

Most MIPs are synthesized in bulk; the resulting polymer is then ground and sieved into the desired particle size range. The particle size ranges between 20 and 90 μm, which is suitable but far from optimal for packing in a chromatographic column. Sieved particles have been packed into conventional HPLC columns,[16,24] immobilized on TLC plates,[25] and used in capillary columns.[26,27] These polymers are appropriate for use in chromatographic applications because of their ability to withstand high pressures, temperatures, extremes in pH, and organic solvents. The ground MIP powders can be kept without any preservatives and can be used for columns packing readily.

Although synthesis of MIPs by bulk polymerization is relatively simple and rapid, the particles obtained by grinding and sieving are polydisperse in both size and shape. The process of grinding and sieving is tedious and cumbersome, and results in substantial loss of polymer; only a small fraction of the polymer recovered is suitable for use as a packing material. The irregular polymer particles limit the efficiency and resolution in terms of chromatographic performance.[11] The efficiency of the column decreases due to peak broadening and severe peak tailing of the analyte recognized and retained in the imprinted polymers.

Previous studies have documented improvements in the shape and size distribution of MIP particles through alternate synthetic processes.[28] Surface imprinting approaches have been investigated to address the problems associated with mass transfer and accessibility.[2] Methods such as suspension,[20a,29] emulsion,[30] precipitation,[31] and dispersion[32,33] polymerization were explored to generate more uniform polymer particles. MIPs generated by the heterogeneous polymerization methods above can be used as a dispersion or can be further processed to form films or coatings and can be deposited on supports of any geometry.

Emulsion polymerization is capable of producing uniform particles with diameters smaller than 1 μm. However, only a limited number of MIP beads have been successfully prepared using this polymerization technique.[2] Dispersion polymerization is another method for preparing MIP beads of 1–10 μm particle size. The polymerization starts with a homogeneous solution of monomer, initiator, and polymeric stabilizer. When the resulting

polymer chain grows to a certain critical point, the polymer precipitates to form spherical particulates. The polymeric stabilizer acts to prevent these particulates from coagulation. This approach has been demonstrated by Sellergren,[32] who prepared porous MIPs in 15 cm × 3 mm i.d. glass tubes using a mixture of isopropanol and water.

Using both aqueous and non-aqueous continuous phases, suspension polymerization has been investigated for the preparation of MIP beads. Beads of 20–200 μm can be generated using this technique. Suspension polymerization starts with a dispersion of droplets containing monomer, initiator, and solvent in a continuous phase. With the aid of an appropriate surfactant, the droplets are maintained by mechanical stirring of the mixture during polymerization. Colloidal polymer particles have also been synthesized via a two-stage emulsion polymerization technique,[34,35] in which a combination of covalent imprinting and non-covalent rebinding were used for creating polymers imprinted with cholesterol.

Most approaches to MIP chromatography reported to date have focused on capillary column[36,37] separations. Packed capillary columns have been used in capillary electrochromatography (CEC) separations as has been demonstrated in numerous papers.[38–40] The preparation of these columns includes the fabrication of retaining frits within a capillary and the packing of small diameter particles into narrow-bore capillaries. However, both these steps present technical difficulties associated with packing and retaining beads in narrow bore columns, and thereby reproducibility of this procedure remains problematic.

These limitations have led to the development of various alternative approaches. Fritless monolithic columns, in particular, which emerged during the last decade,[27,37,41–47] have proven to be a viable alternative to packed capillaries. These are prepared by in situ polymerization to form a continuous porous bed. The monolithic format is an attractive approach for preparing MIP stationary phases. With this format, laborious packing procedures, such as frit making as well as bulk polymerization of MIP with subsequent crushing and sieving steps, are avoided. In addition, monolithic columns have several advantages,[48–50] which include: (a) the polymerization process is simple and can be performed directly within the confines of a capillary or a microfluidic chip, thus avoiding the problems related to both frit formation and packing; (b) columns of virtually any length and shape can be fabricated; (c) the polymerization mixture can be prepared using a wide variety of monomers, allowing a nearly unlimited choice of both matrix and surface chemistries; and (d) the polymerization process can be easily controlled, which enables optimization of the porous properties of the monolith, and consequently the flow rate and chromatographic efficiency of the system.

Sellergren reported the first use of an MIP as a stationary phase in a capillary column; the sorbent was synthesized in situ by dispersion polymerization, which involved the formation of about 10 μm sized agglomerates of 0.5–4 μm particles.[36] These MIP capillaries were imprinted

against L-PA, pentamidine, and benzamidine. The results showed low selectivity of these columns and only pentamidine showed a pH-dependent retention that might originate from imprinting. Despite the poor performance of these columns, this work is of high importance in indicating the possibility to synthesize MIPs in situ.

Porous monoliths of MIPs have also been prepared in situ in 75 μm i.d. × 35 cm long fused-silica capillaries using a non-polar solvent (toluene) with UV initiation at −20°C.[37] Columns created from this study were able to separate a racemic mixture of the β-adrenergic antagonist propranolol in the CEC mode.

MIP capillary columns can also be prepared as thin films for open-tubular liquid chromatography (LC)[11] using in situ thermal polymerization inside 25 μm i.d. fused-silica capillaries. The columns generated low flow resistance, which enabled separations at very low pressure (<1 bar/m of column length). Chiral separations of dansyl-D L-phenylalanine were achieved using both open-tubular LC and open-tubular CEC.

A. Combinatorial Approaches

Though polymer preparation by non-covalent imprinting is relatively simple and rapid, most of these procedures were never optimized. Potential problems, which could occur resulting from non-optimized procedures, include low binding specificity, template leaching, slow kinetics, and low affinity. Therefore, the development of methods to optimize the main factors affecting the molecular recognition process is required. This might best involve a combinatorial chemistry based approach, combining the MIP technique and the combinatorial chemistry model to allow rapid screening of combinatorial libraries of MIPs to identify those with the desired levels of capacity and selectivity for a given target molecule.

This technique has been studied in detail by Takeuchi et al.[22] for the combinatorial synthesis of MIPs. A library of MIPs was prepared and screened in a semi-automated system using a programmed liquid handling equipment. Mini-MIPs were prepared in situ on the bottom of individual glass vials, followed by a release and rebinding test of the template molecule in the polymer matrix. In this work, libraries of MIPs, templated against a triazine herbicide, ametryn or atrazine, were prepared using various amounts of two functional monomers, MAA and 2-(trifluoromethyl) acrylic acid (TFMAA). The results indicate that the combinatorial approach is a promising method for identifying the optimal conditions for MIP preparation for a given molecule. Due to the low consumption of reagents, the small-scale protocol is well suited for automation of combinatorial studies.[22]

Most MIPs synthesized by the non-covalent approach are prepared and analyzed in non-polar solvent systems. Under these conditions, hydrogen bonding often plays a major role in the recognition process between the template and the monomers. In one study, dipeptide N-Ac-Phe-Trp-Ome

was imprinted with chloroform as the solvent.[51] The imprinted sites and binding capacity of the imprinted polymer were then evaluated with solvents with different polarity. Their results showed that the selectivity of the polymer was higher when a less polar solvent such as chloroform is used than a more polar solvent such as acetonitrile. It was also shown that the use of aqueous mobile phase reduces the selectivity of the template selectivity due to the reduced hydrogen bonding interaction between the template and the polymer.[52,53]

Other studies have shown that ion exchange and hydrophobic interaction effects are more significant than hydrogen bonding in a highly aqueous mobile phase, but may also contribute to non-specific interactions between the template molecule and the polymers.[54,63] Five structurally related triazine herbicides with various hydrophobicity and basicity were imprinted. The results of this study showed that selectivity of molecular imprinting in aqueous rich mobile phase correlates to the hydrophobicity of the template. Depending on the hydrophobicity of the template, selectivity in the rebinding step is enhanced by increasing the amount of aqueous media in the mobile phase.

After preparing the polymers according to the steps described above or in the imprinting recipes from other literature examples, the imprinting effect of the MIP must be evaluated to determine whether the imprinting protocol successfully produced any specific recognition sites. Figure 8 can be followed to demonstrate the effectiveness of the imprinting effect of the newly prepared MIP. Binding strength of the MIP is compared with a control polymer, a non-imprinted polymer prepared exactly the same as the MIP, but in the absence of the template. Another control polymer, MIPx is also prepared in exactly the

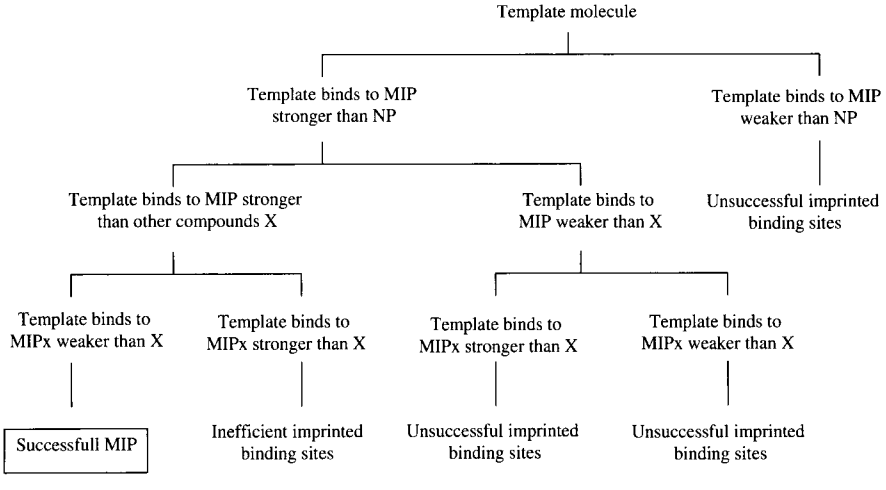


FIGURE 8 Flowchart describing means of achieving the imprinting effect.

same way as the MIP, but with a compound that is structurally related to the template.[2]

IV. USE OF IMPRINTED POLYMERS IN SEPARATION SCIENCE

The increasing demand for greater pharmaceutical and environmental analysis throughput requires analytical chemists to look for new ways to improve analytical procedures with better selectivity and sensitivity. Recently, the use of selective MIPs as an analytical technique has gained interests as a novel type of sorbent with attractive properties. MIPs have been applied in many analytical techniques, including chromatography (LC, CE, and CEC), SPE, ligand binding assay, and sensor technology. Below we discuss selected MIP applications with a focus on analytical separations.

A. Liquid Chromatography

MIPs have been used extensively as stationary phases for LC as can be seen from several reviews.[55,56] Most of their applications include he use of MIPs for chromatographic separations since MIPs can be custom-made with selectivity for a pre-determined molecular species. This is very advantageous, for example, for enantiomer separations as the isomer used for the preparation of MIP is always the one that is more strongly retained. As a result, the elution order is often predictable as opposed to using commercial chiral stationary phases (CSPs) in which further measurements would be necessary for rapid identification of the analytes. Separations of enantiomers have been achieved, for example, for drug compounds,[57–60] amino acid derivatives,[16,55] and sugars.[5] MIPs have also been applied to chromatographic separations of natural products, pharmaceuticals, clinical, and environmental compounds using LC.[61–70]

In practice, LC has been the most widely used method for qualitatively and quantitatively assessing MIP efficacy. MIPs are mostly used as HPLC sorbents in packed columns, in which effective separations can be achieved due to the high selectivity of the stationary phases. Column efficiencies, however, are generally lower compared with using commercial stationary phases. Another limiting factor in performance of these columns is extensive peak broadening and tailing, which is present more often than not, and is especially problematic for the more retained compound (usually the template). This problem often arises when MIPs are prepared as bulk polymers, and then ground and sieved before being packed into LC columns. It has been reported that the drawback is due to the heterogeneity of binding sites, in terms of both affinity and accessibility, and different association and dissociation kinetics.[71,72] Further improvements in efficiency have resulted from the development of novel suspension polymerization techniques,[30,73] two-step swelling techniques,[74,75] open-tubular LC,[51] and in situ synthesis.

B. Capillary Electrochromatography

CEC is a hybrid method between capillary HPLC and capillary electrophoresis (CE). It combines the high separation efficiency that capillary zone electrophoresis (CZE) offers with the wide range of parameters that HPLC offers, particularly with a wide range of stationary phases to choose from. Subsequently, CEC has become a powerful technique that has gained considerable interest in the last few years.

The use of MIP sorbents for CEC is of great interest, largely due to the need for a highly effective liquid phase separation system with unique selectivity for pre-determined molecular species in a miniaturized format. This is especially attractive since CEC is known to yield greater efficiency than conventional HPLC, and thus CEC-mode separations should lead to an improved performance of imprinted polymers compared with that achieved in conventional LC. Another advantage over LC is that the capillary format leads to minimal consumption of reagents and analytes so that commercial scale production of MIP-based micro-columns for CEC is achievable.[17]

The first requirement for MIP-CEC is therefore the adaptation of the molecular imprinting technology to the capillary format. This is not a simple task and the straightforward approach taken from HPLC experiments by packing irregular MIP particles to prepare MIP sorbents presents some technical difficulties in the capillary format. Several studies that have been conducted and reviewed,[56,76] mostly focused on how to synthesize MIP stationary phases within fused silica capillaries. Several approaches have been reported on how MIP stationary phases can be employed in the capillary format: immobilized inside capillaries using a polyacrylamide gel,[26] in situ dispersion polymerization,[36] monolithic super porous imprinted polymers prepared by an in situ photo-initiated polymerization,[37,77] and thin film coatings of MIPs in open tubular CEC.[78] To date, the monolithic format seems the most attractive approach to obtain capillary columns with an MIP stationary phase by in situ synthesis. Monolithic columns have special characteristics and present several advantages, mainly in the ease of their preparation. With this format, the polymerization process is performed directly within the confines of a capillary, thus avoiding the problems related to both frit formation and packing.

C. Solid-Phase Extraction

MIPs have also been applied to SPE (MIP-SPE), mostly for biological and environmental samples.[62,63] The use of MIPs as SPE sorbents is the most practical application of MIPs, and therefore, it has been widely studied. This technique is advantageous for efficient sample clean-up given the pre-determined selectivity of MIPs, which is governed by the choice of the template, combined with the high selectivity of the sorbent. The first study on MIP-SPE was sample enrichment of a spiked urine sample[65] employing

an MIP selective for pentamidine. The use of an MIP in an SPE device is especially important in trace analysis of environmental samples due to the capability of MIP to extract larger sample volumes, thus improving sensitivity. Because of the irregular shape and size dispersity of the MIP particles, low chromatographic efficiency resulting from peak broadening was observed. This is a major limitation of MIPs as chromatographic separation media, but much less of an issue for SPE, since SPE is less sensitive to the poor chromatographic performance of the sorbent.

Many different target analytes have been extracted from a variety of matrices using MIP sorbents in the SPE format, including various biological fluids in neat, diluted or protein-precipitated form,[79–84] and organic solvent extracts of water samples,[70,71] biological tissues and fluids,[85–88] chewing gum,[89] tobacco,[90] and fermentation broth.[91] Several modes have been reported for MIP-SPE: on-line SPE,[79,87,90,91] conventional SPE (MIP is packed into columns or cartridges),[80–84,92–96] and batch mode SPE (MIP is incubated with the sample).[88]

MIP polymers also tend to swell when the solvent composition is altered,[17] which often leads to irreversible deformation of the imprinted cavity and loss of selectivity. Better understanding of the MIP imprinting process and the recognition mechanism will provide improved imprinting technique and enhanced chromatographic separations.

V. PHARMACEUTICAL APPLICATIONS OF MIPs

The pharmaceuticals industry continues to invest heavily in research and development for the production of novel drug substances. The demand of this industry for rapid and effective analytical strategies that drive improvements in the quality of their products results in a constant search for new analytical methods. This section will discuss the potential roles for molecularly imprinted polymers in the pharmaceutical sciences. The advantages of MIPs, e.g. physical robustness, resistance to high pressure and temperature, and tolerance of different solvents and media, have led to modest increase in their use in the pharmaceuticals sector. MIPs have been implemented in various applications including sample preparation, as stationary phases for analytical separations, and as analyte recognition materials in affinity assays.[97]

SPE based on MIPs is a novel approach for sample preparation and preconcentration. The implementation of MIPs in SPE devices for the separation and detection of drugs and drug metabolites has great potential in the pharmaceutical industry. The control of pharmaceutical impurities is a critical issue. Impurities in pharmaceuticals are the unwanted components that remain with the active pharmaceutical ingredients (APIs), or develop during formulation, or upon aging of both API and formulated APIs. The presence of these unwanted components may influence the efficacy and safety of

pharmaceutical products. As a consequence of these considerations, the International Conference on Harmonization (ICH) has issued guidelines on the levels of impurities and degradation products that should be determined in drug substances and drug products as well as guidance for analytical approaches to assure that test methods can meet these criteria.[98–101] This guidance has been adopted by the US Food and Drug Administration (FDA) and other regulatory authorities throughout the world.

HPLC methods applicable to the analysis of drug substances/drug products should be able to separate the API from the impurities and degradation products. Analysis of these trace amounts of impurities in the presence of a large quantity of API is problematic in particular because the impurities are usually structurally related to the API. The use of imprinted polymers as separation media for drug substance and drug product analyses is particularly important for the isolation of degradation products and impurities from the API. Such isolation will allow for impurity profiling in the absence of APIs, and therefore, assure that the analytical method is specific.

The setback associated with MIP-SPE is the presence of unremoved template in the polymer matrix. This means there are fewer binding sites that are available for the analyte, which reduces the column capacity. This limitation is much less an issue in trace analysis, however, where the sustained template leakage can interfere with the detection and the analysis of the analyte. This problem can be overcome by using an analogue to the imprint molecule in the synthesis. By using a structural analogue to create the MIP sorbents, selective binding of the analyte is achieved with SPE.[102]

The employment of MIPs as synthetic molecularly selective artificial receptors has a broad potential base of application in many areas of the pharmaceuticals industry. The superiority of MIP-based affinity assays over the traditional antibody-based approach include the greater ease, reduced cost, and shorter time involved in preparing the MIP, its physical robustness, mechanical, and thermal stability compared with antibodies.[97] An MIP created by imprinting a specific ligand can be used to screen a library of compounds with binding strength close to the imprinted ligand by using the MIP as stationary phase in an HPLC setup. This approach can assist in the screening of alternative substances that bind to the biological receptor of the known ligand. This is especially important when the biological receptor itself is not readily available.[103]

MIPs have been used in the area of drug delivery and targeting. They have been used as materials to allow controlled delivery of drugs to ensure that drugs are released to target site at desired dose and time in order to enhance its pharmacological profile and minimize any side effects. As part of MIPs as drug delivery systems, a number of approaches have been developed and reviewed,[104] such as rate-programmed drug delivery, activation-modulated drug delivery, and feedback-regulated drug delivery. In each case, the rate of drug release is either regulated by a specific rate profile;

activated by some physical, chemical, or biochemical processes; or by the level or concentration of a triggering agent such as a biochemical agent.

All these approaches have shown the applicability of MIPs as tools to modify the release profile of a drug from a polymer matrix. Hiratani[103,105] has demonstrated new imprinting technology using imprinted hydrogels from soft contact lenses imprinted with timolol maleate for ophthalmic delivery. Initially, the influence of hydrophilic monomers on the affinity of the lenses toward timolol was studied. *N,N*-diethylacrylamide (DEAA) lenses showed the greatest imprinting effect, while (2-hydroxyethyl) methacrylate (HEMA)-based polymers showed the highest affinity for timolol, which was due to increased capacity of HEMA to hydrogen bond with timolol. Moreover, a much longer duration of timolol release for the imprinted lenses was maintained compared with non-imprinted lenses. The longer duration of timolol release was due to improved capacity of timolol with respect to a non-imprinted polymer.

A few cases were reported in which MIPs were used for a sustained release of a drug and additionally an enantioselective release. In the sustained release, the imprinted drug was released slowly from the matrix because of its high affinity to the MIP. An example was a study conducted by Allender et al. with the release of propanolol from the propanolol-imprinted transdermal delivery system.[106] An MIP imprinted with an enantiomer successfully retained the same enantiomer in a matrix loaded with the racemic drug, resulting in its slower release rate compared with the opposite enantiomer, which did not fit in the binding site cavities. Another useful application of an MIP in drug delivery is responsive drug release by competitive binding. This is accomplished when a weakly bound compound was released after stimulation by binding of the imprinted compound to the MIP. This concept was first introduced by Sreenivasan.[107] The study showed the successful preparation of hydrocortisone-imprinted polymers and its application as responsive release system by slowing the release of testosterone induced by hydrocortisone from hydrocortisone-imprinted polymers.

The use of MIPs as components of drug targeting devices can be achieved by producing a synthetic drug targeting element, designed by creating imprints of the targets that are exposed on cell or tissue surfaces. The drug, coupled covalently or non-covalently to the MIP, is released when the MIP binds to its target on the surface of the cell.[108,109]

VI. PROS AND CONS OF MIP SORBENTS

The MIP-based approach to high selectivity offers several important advantages, including the ability to tolerate high pressures, organic solvents, pH extremes, and elevated temperatures.[110] The high degree of cross-linking of MIP polymers provides unique chemical, mechanical, and

thermal long-term stability compared with their biological counterparts. The materials used for synthesizing MIPs are readily available and inexpensive, with the possible exception of the template. In both covalent and non-covalent imprinting approaches, the template can be recovered and reused, which may be particularly important when the availability of the template is low and/or it is expensive. Preparation of MIPs can easily be scaled up, especially when the non-covalent approach is employed.

It is evident that there are some problems associated with the use of MIPs in chromatographic separations under isocratic conditions, which usually results in a sharp peak corresponding to the weakly retained analyte and a broader peak and severe tailing corresponding to the template molecule. This behavior of MIP sorbents is often attributed to the unfavorable adsorption isotherm and slow mass transfer in the polymer matrix, two of the major limitations of MIPs as separations media. Because the arrangement of the monomers around the template is not specific, the binding sites inside the polymer matrix have a range of affinities toward the template. The difference in binding strength of various imprinted sites gives rise to a non-linear adsorption isotherm that results in extreme tailing of the more retained peak.[110–112] These effects can be minimized by optimizing the chromatographic conditions by using gradient elution.

VII. CONCLUSIONS AND FUTURE OUTLOOK

Despite significant progress in methods of synthesis of MIPs and notable development of new applications for MIPs, it remains necessary to establish new and better techniques, applications, materials and synthetic methods for molecular imprinting to optimize the potential of MIP sorbents. Thus, so far, the majority of MIP applications have focused on low molecular mass analytes. Only a limited number of studies have been done on the imprinting of macromolecules such as proteins. In addition, most MIP syntheses and evaluations are performed in organic solvents. Finding new ways to prepare MIPs that perform efficiently in aqueous systems, such as serum, plasma, and ground water, is desirable. Development of methods for the production of MIPs in aqueous media may open up the possibility of making imprints of very labile molecules, such as peptide drugs and oligonucleotides. Interest in molecular imprinting technology is on the rise, especially in the field of pharmaceuticals research. Significant progress is likely in this field, one major area of study being geared toward enhancement of applicability of MIPs for drug delivery. Combinatorial approaches to MIP synthesis in the future will allow for rapid screening of candidate MIPs to allow selection of sorbents that exhibit desired levels of capacity and selectivity for a given target molecule, thus brightening the future of imprinted polymers as media for liquid-phase separations.

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