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Loralie J. Langman
Christine L.H. Snozek
Editors

LC-MS in Drug Analysis

Methods and Protocols

Second Edition

Humana Press

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Preface

The second edition of this book is again intended to provide detailed LC-MS(/MS) procedures for the analysis of compounds of clinical significance. The main focus points for this edition were new developments including novel drugs (both therapeutic and recreational) and updated methodologies, as well as discussing alternate matrices not addressed in the first edition.

We thank our colleagues who contributed to the contents of the book for the countless hours of work that these chapters represent. We hope that you, the reader, find this book useful.

Rochester, MN, USA — *Loralie J. Langman*
Scottsdale, AZ, USA — *Christine L.H. Snozek*

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Chapter 1

An Introduction to Drug Testing: The Expanding Role of Mass Spectrometry

Christine L.H. Snozek, Loralie J. Langman, and Steven W. Cotten

Abstract

Measurement of drugs and their metabolites in biological fluids is the foundation of both therapeutic drug monitoring (TDM) and toxicology. The introduction of methods based on mass spectrometry (MS), coupled with gas or liquid chromatography, has revolutionized these areas. This chapter will introduce the reader to the application of MS to TDM and toxicology, the steps that should be considered during implementation and the processes that should be implemented to assure continued quality. Points of emphasis include advances and recent trends since the publication of the first edition of this book, such as high-resolution mass spectrometry and increased interest in alternate matrices.

Key words Mass spectrometry, Gas chromatography, Liquid chromatography, Therapeutic drug monitoring, Toxicology, Drug testing

1 Introduction

Measurement of drugs and their metabolites in biological fluids is the foundation of both therapeutic drug monitoring (TDM) and toxicology. Though different in their application, each of these disciplines depends upon accurate identification and quantification if drug measurements are to be useful. Thousands of methods are described for drug analysis, but until recently most have relied upon analytical tools that suffer from lack of specificity and sensitivity, namely, spectrophotometry and immunoassays. It must be acknowledged that the methods utilizing each of these allowed TDM and toxicology to grow and mature, but it is mass spectrometry that is taking the analysis of drugs and drug metabolites into new directions.

In recent years, mass spectrometry whether single stage, tandem (MS/MS), or high resolution (HRMS), coupled with liquid chromatography (LC) or gas chromatography (GC), has emerged as a powerful tool for clinical and toxicology laboratories.

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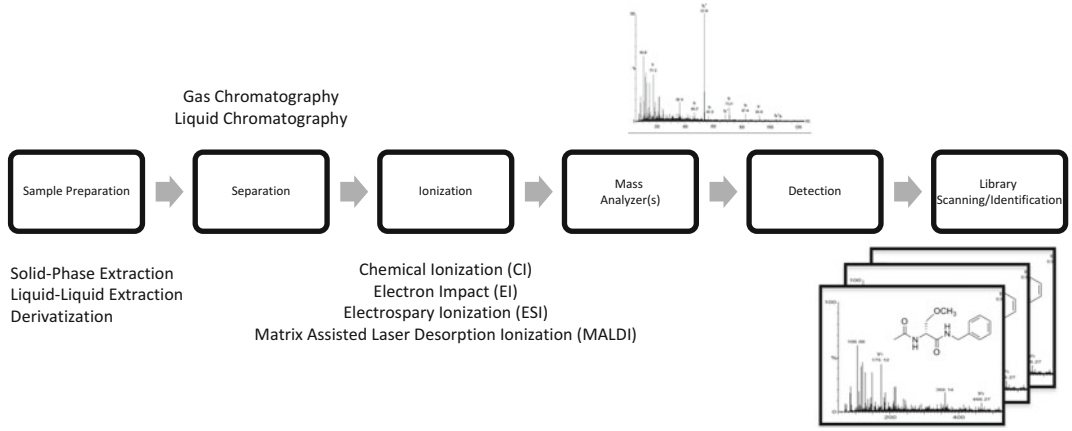

Fig. 1 Current applications of mass spectrometry in the clinical laboratory

Improvements in user interfaces, computing power, and column characteristics have expanded the potential of MS-based systems from rigid and cumbersome techniques to limitless, adaptable open-source platforms capable of identifying numerous analytes in a single sample. The sensitivity and accuracy achievable have naturally found applications in the areas of TDM and toxicological analysis and beyond (Fig. 1). Evidence of the transition to the clinical setting is seen not only by the number of publications but also in the development of quality management guidelines and standards [1, 2].

This volume will provide the reader with a number of applications to both disciplines which will be introduced in the following pages. This chapter will also discuss some of the issues one should consider when migrating to MS-based methods. The emphasis of this second edition is on newer drugs (e.g., designer drugs of abuse) and technologies that have emerged since the first edition, as well as a focus on alternate matrices beyond traditional blood and urine specimens.

2 Therapeutic Drug Monitoring

Therapeutic drug monitoring is an integral part of personalized medicine. By providing accurate quantification of drug concentrations in the circulation using blood, serum, or plasma, TDM is used to maximize the effect of certain prescribed drugs by achieving a therapeutic concentration as quickly as possible while simultaneously minimizing unwanted or toxic side effects. The drugs typically monitored are those with narrow therapeutic indices and for which there are established relationships between the concentration found within the circulation and the observed effects of the drug. For these drugs, the delicate balance between efficacy (ED_{50})

and toxicity (LD_{50}) dictates the need for accurate quantification. TDM also provides a means of assessing compliance, ensuring correct dosing, and identifying drug-drug interactions. Ironically TDM initially developed in parallel with the introduction of chromatography-based methods into the clinical laboratory in the early 1970s.

Unfortunately, the methods were time-consuming and in the 1980s were replaced with immunoassay-based methods which remain in use in many laboratories today. While these methods offer advantages of ease of use and availability on many analytical platforms, they suffer from issues of sensitivity and specificity. Positive and negative interferences are well documented and if not recognized can lead to inappropriate patient care. In addition, these methods usually cross-react with structurally related metabolites which may or may not contribute to the pharmacological activity of the parent drug. Other compounds which also share structural features with the drug being measured are also likely to cross-react with the antibody, and so the presence of such compounds also poses problems. Finally, the limit of detection of many immunoassays is insufficient for current TDM applications. For example, evaluation of methotrexate clearance uses low micromolar cutoffs, at a concentration range where immunoassay cross-reactivity with metabolites becomes a significant concern. Clinical laboratories have thus replaced many of the TDM analyses using immunoassay with methods using LC-MS/MS [3–7].

Monitoring of immunosuppressant therapy for solid organ transplant patients using LC-MS/MS was perhaps one of the first applications that adopted MS analysis. Subtherapeutic doses can result in transplant rejection, while overdosing can cause serious toxicity or death. It is therefore imperative to closely monitor individual patient drug levels for proper treatment. LC-MS/MS permits simultaneous quantification of immunosuppressant drugs from one whole blood sample [8–10]. Whole blood samples are lysed and precipitated (either offline or online) followed by mass analysis with internal standards. Comparisons of LC-MS/MS with immunoassays for immunosuppressants often demonstrate lower drug concentrations in LC-MS/MS assays [9], with the difference being attributed to the continued issue of cross-reactivity of immunoassay antibodies with drug metabolites.

A relatively new arena for TDM opened with the introduction of monoclonal antibody (mAb) therapies, used to treat a variety of conditions including Crohn's disease and ulcerative colitis. These biologic agents provide the ability to target specific aspects of the immune system, to modulate immune response against self- and foreign antigens. Measurement of mAb concentrations gives some indication of likelihood of successful therapy and can aid in distinguishing subtherapeutic concentrations from nonresponsiveness to treatment. However, many of the mAb therapies are themselves

immunogenic, necessitating simultaneous measurement of the mAb and neutralizing antibodies that block the activity of the drug [11].

The aforementioned drugs or classes represent but a few of those for which LC-MS/MS methods have been described. Methods for antiepileptic, antidepressant, antimicrobial, and chemotherapeutic agents are readily found, with some being the only methods described. Clearly another advantage in the adoption of MS techniques is the ability to develop assays in-house rather than having to wait for immunoassay manufacturers.

3 Toxicology

Originally the "study of poisons," toxicology has evolved into a broader, highly diverse field. Today we recognize that poisons are readily found in the home, work, and environment, can be man-made or naturally occurring, and may be therapeutic in other circumstances. Unfortunately, toxicity may not become apparent until after the offending agent is metabolized or even cleared by the body. This poses an interesting challenge. As with TDM, the use of mass spectrometry-based methods has extended the testing range and may, in the near future, facilitate discovery long after a toxin is gone. Still in the early stages and not quite ready for clinical or forensic application, proteomic and metabolomic profiling using mass spectrometry have revealed patterns that one day will likely be used to identify toxins months or years after an exposure.

In the clinical setting, broad screens seeking offending agents were largely abandoned in the 1990s, again primarily due to the lengthy times required to complete the analyses and the change in focus to drugs of abuse. Broad screens are not high-volume tests, but there are clinical situations in which such a test is useful in excluding a toxin as a cause of the patient's symptoms. One such example is highlighted by the emergence over the last decade of designer drugs, e.g., synthetic cannabinoids, cathinones, and fentanyl analogs. The rapid evolution of new psychoactive substances (NPS), in efforts to evade detection and legislation, has created intense interest in technologies such as high-resolution mass spectrometry (HRMS) which can tentatively identify novel compounds without a spectral library and before standard reference material is available. HRMS complements existing technologies but may eventually supplant GC-MS-based screening as the preferred choice for small molecule identification. Confirmation against a spectral library or reference standard can be done for known compounds as with conventional screens; for emerging compounds, HRMS data can be acquired in an untargeted manner and reanalyzed at a later date [12].

For many years, GC-MS was considered the gold standard for confirmation of the presence of abused drugs in urine, but as methods and libraries have developed, many laboratories have turned to LC-MS/MS for these analyses. In the instance of pain management, screening and confirmatory drug testing using LC-MS/MS allow for detection of both morphine-based and synthetic opioids. The technique provides superior sensitivity and specificity compared to immunoassays which are usually targeted to morphine and may thus fail to detect synthetic opioids and oxycodone. The ability to detect multiple parent drugs and metabolites in a relatively short run makes LC-MS/MS a powerful tool in support of pain management testing. The use of this technique also provides an extended analytical measuring range of approximately 10^5 ng/mL that is most useful in this setting [13].

Matrices other than blood and urine are an increasingly important component of clinical and forensic toxicology. Meconium and umbilical cord tissue can reveal in utero exposure to illicit drugs. Oral fluid, hair, and dried blood spots all provide certain advantages over traditional analytical matrices. For example, oral fluid testing can allow for observed collection of a specimen during a roadside stop from a seemingly intoxicated driver, while dried blood spots can facilitate testing collected in remote locations far from a reference laboratory.

4 Evaluation of Methods

A detailed discussion of chromatographic methods is beyond the scope of this book, though some features will be discussed in this section. Generally, these techniques are used to separate the drugs of interest from other compounds present in the sample. Afterward the analyte is introduced into the mass spectrometer which serves as the detector. Which chromatographic technique is used depends upon the sample and the volatility and solubility of the target analyte.

Depending upon the biological matrix and target compounds being analyzed, pre-analytical treatment of the sample is generally necessary as seen in Fig. 2. Removal of interfering components such as proteins and lipids improves sensitivity by decreasing the complexity of the mixture analyzed. Liquid-liquid or solid-liquid extraction may be necessary prior to introduction on the chromatography system to enrich for target compounds. For compounds present in low abundance, extraction followed by evaporation of the solvent to dryness effectively concentrates the sample, improving sensitivity. For relatively abundant compounds, a simple deproteinization step (i.e., the dilute and shoot method) may suffice. In this, the sample is mixed with an organic compound such as acetonitrile (spiked with the appropriate internal standard) and

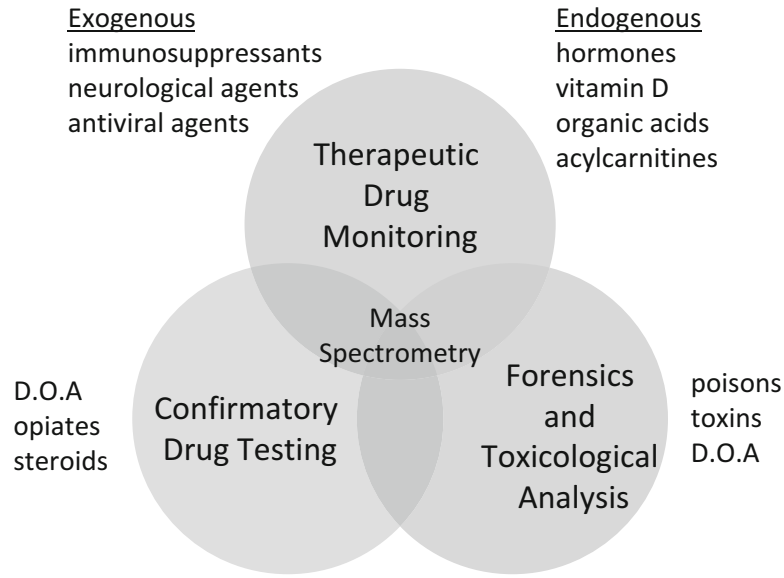

Fig. 2 Pipeline of steps involved in mass spectrometry analyses

centrifuged, and the supernatant is injected. These methods work fairly well for many LC-MS- or LC-MS-/MS-based methods.

Internal standards should be added at the beginning of analysis as both a quality control measure and to facilitate quantification. Where possible, it is recommended that analogs labeled with a stable isotope, e.g., deuterium or ^{13}C, of the primary analyte of interest be used as the internal standard. If such is not available, it is acceptable to use a compound that is structurally related. The internal standard must undergo all steps of the procedure (extraction, derivatization, evaporation, etc.) in order to serve the purpose of identifying problems that could arise during the sample preparation. Since small amounts of the unlabeled compound may contaminate the internal standard, it should be checked by analyzing a blank sample to which the internal standard is added.

It is usually necessary to separate the analytes of interest from each other and from unrelated compounds by the use of a column. In liquid chromatography, gradient solvent systems of methanol, water, or acetonitrile (or mixtures thereof) are frequently used to sequentially elute compounds based on polarity and affinity for the column. As laboratorians face increasing work demands, further simplification of methods by direct introduction of the sample into the mass spectrometer has been explored and continues to gain in popularity. Online sample extraction, for example, by turbulent flow chromatography (e.g., Thermo Cohesive), or high-throughput solid phase extraction (e.g., Agilent RapidFire), can greatly facilitate assay throughput by reducing analytical run time and/or allowing multiplexed analysis.

After separation, the compounds are ionized prior to mass analysis. Charged compounds are shuttled into the mass analyzer which, depending upon the method, selects ions based on predetermined mass-to-charge ratio (m/z) criteria or scans within defined m/z ranges. If tandem mass spectrometry (MS/MS) is used, the selected ionized compounds are further fragmented followed by an additional m/z detection to obtain spectra for both precursor (formerly known as "parent") and product (formerly known as "daughter") ions. The data are transformed into a recognizable mass spectrum which, in conventional mass spectrometry, is subsequently compared to expected values for the target compounds or internal standards for quantification or to a library of chemical spectra to obtain the identity of the compounds analyzed. Untargeted HRMS permits tentative identification even in the absence of a known spectral library.

5 Quality Assurance

Each laboratory should develop a quality assurance program based upon their respective regulatory guidelines and needs. Such programs provide guidance to the analyst regarding method validation and maintenance with sufficient checks to assure that the results reported are as accurate as possible. Table 1 provides a list of documents that may be useful in developing a robust quality assurance program.

Method validation should include assessment of the limit of detection (LOD), limit of quantification (LOQ), linearity, selectivity, accuracy, precision, carryover, matrix effects, and reference intervals. For those drugs and analytes regulated under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, the laboratory should use the mandatory precision limits to targeted day-to-day precision necessary for successful proficiency testing [14]. Alternatively, one should consider the application of the

Table 1
CLSI documents relevant to mass spectrometry analysis

Useful resources and documents for quality assurance programs	
CLSI C43-A2	Gas chromatography-mass spectrometry confirmation of drugs
CLSI C50	Mass spectrometry in the clinical laboratory
CLSI EP 05	Evaluation of precision performance of quantitative measurement methods
CLSI EP 06	Evaluation of the linearity of quantitative measurement methods
CLSI EP09	Method comparison and bias estimation using patient samples
CLSI C24	Assessment of laboratory tests when proficiency testing is not available

analysis when setting precision and accuracy goals. If TDM is the application, the actual precision and accuracy needs may exceed those of CLIA.

Each analytical run should include an adequate number of quality control samples containing the targeted analytes or drugs considered representative of those expected. Concentrations should target decision points and span the analytical measuring range. For example, a method used for TDM of a drug should include control samples below, within, and above the therapeutic range. Assays frequently used to monitor clearance, e.g., leflunomide and methotrexate, should include a quality control near the clinical decision limit or the LOQ, as appropriate. A method used for confirmation of the presence of an abused drug should include control samples in which the drug is absent and present near and above the defined cutoff. Blind quality control samples may be included to assess the nonanalytical portions of the entire process. Proficiency testing is also an important part of quality assurance. Clinical laboratories operating under CLIA must have control compounds for both quantitative and qualitative confirmatory drug testing and control compounds for each drug class surveyed in broad-spectrum screening using GC-MS and must participate in proficiency testing for each analyte reported [14]. In these challenges, the proficiency samples are tested as ordinary samples. The development of new methods for various drugs is often ahead of the availability of commercial sources of such samples. In these cases, it is reasonable for several laboratories performing the analysis to exchange samples on a regular basis (at a minimum of twice per year).

Forensic laboratories in the United States still face some issues related to lack of standardization and accreditation [15, 16]. Some of the outlined challenges regarding standardization and proficiency related to analyte identification should be addressed through the development and adoption of standards of drugs and drug metabolites suitable for mass spectrometry. For example, national and international authorities have published guidelines for drug testing that include mass spectrometry as the premier analytical method for unambiguous analyte identification [17, 18]. However, further work remains to be done in this arena.

6 Conclusions

Recent years have seen expansion of the importance of mass spectrometry to TDM and toxicology. TDM has expanded from its historical arena of small-molecule agents to embrace proteolysis-aided quantitation of mAb therapies. HRMS and LC-MS/MS have rapidly risen to the forefront of efforts to detect and identify a growing and ever-changing list of NPS. The ability to

retrospectively identify compounds seen during untargeted acquisition on HRMS has allowed better epidemiological tracking of issues related to NPS such as outbreaks of toxicity after exposure to a new agent or contaminated batch of drug.

As separation techniques continue to improve and hardware and software platforms advance, the role of mass spectrometry in the clinical lab will continue to grow. When evaluating a new mass spectrometry method, the concepts of linearity, sensitivity, specificity, accuracy, and precision should be at the forefront. Proper validation will ensure that the quality of the diagnostic data provided remains high with the improved fidelity afforded by mass spectrometry.

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Chapter 2

Quantification of Eight Cannabinoids Including Cannabidiol in Human Urine Via Liquid Chromatography Tandem Mass Spectrometry

Karl B. Scheidweiler and Allan J. Barnes

Abstract

Medical and recreational cannabis legalization has highlighted the importance of being able to identify recent cannabis use and impairment. Monitoring minor plant cannabinoids has been proposed to assist in identifying recent cannabis use. Additionally, cannabidiol (CBD) has been proposed for epilepsy, pain, inflammatory disorder, anxiety, and addiction treatment; therefore, monitoring CBD is of increasing clinical importance. However, few methods exist capable of monitoring extensive panels of traditional cannabinoid analytes and minor cannabinoids (including CBD). This chapter details a liquid chromatography tandem mass spectrometry method capable of measuring Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, cannabinol, cannabigerol, tetrahydrocannabivarin (THCV), and its metabolite, 11-nor-9-carboxy-THCV, in urine.

Key words Cannabinoids, Cannabidiol, Urine, Liquid chromatography, Mass spectrometry, Pipette tip extraction

1 Introduction

Interest in monitoring cannabis use and abuse has increased in light of the recent and ongoing legislation regarding medical and recreational cannabis [1, 2]. Δ9-Tetrahydrocannabinol (THC), the psychoactive component of cannabis, is highly lipophilic yielding prolonged excretion into blood and urine after cessation of cannabis use, confounding distinction between recent use and residual excretion post-cessation [3]. Therefore monitoring minor cannabinoids present in cannabis and their metabolites has been proposed for possibly assisting in distinguishing recent use from prolonged excretion. Several minor cannabinoids, such as cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), tetrahydrocannabivarin (THCV), and its metabolite, 11-nor-9-carboxy-THCV

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(THCVCOOH), were proposed as possible markers of recent cannabis intake in different biological matrices [4–8].

CBD is a plant cannabinoid present in cannabis that binds CB2 cannabinoid receptors and has been proposed to be clinically useful for treating many clinical syndromes including epilepsy, pain, inflammatory disorders, and anxiety [9, 10]. CBD also has been studied for treating cannabis and opioid addiction [11]. Therefore, methods capable of monitoring CBD are of increasing interest.

THC is rapidly metabolized to its equipotent metabolite 11-hydroxy-THC (11-OH-THC) and further to its main inactive metabolite, 11-nor-9-carboxy-THC (THCCOOH). All three undergo glucuronidation, are excreted into urine, and are traditional cannabis testing analytical target analytes. Urine cannabinoid testing for multiple cannabinoid compounds and their metabolites presents several challenges, mainly that cannabinoids are extensively glucuronidated requiring synthesis of glucuronide reference standards and/or hydrolysis [12]. Glucuronide reference standards are currently available only for THC and THCCOOH; thus, hydrolysis is required to be able to effectively monitor additional cannabinoids in urine. It previously has been reported that only the THCCOOH-glucuronide ester linkage is easily cleaved via alkaline hydrolysis, while the ether-linked THC-glucuronide bond is not [12]. Therefore previous methods targeting both THC and THCCOOH in urine required tandem hydrolysis via enzyme glucuronidase followed by alkaline hydrolysis [12] or directly monitoring both the free and glucuronidated forms of THC and THCCOOH [13, 14]. Similar research found that CBD was also extensively glucuronidated in urine, requiring enzymatic hydrolysis before analysis, since CBD-glucuronide reference standards are not commercially available [5]. This chapter details a liquid chromatography tandem mass spectrometry (LC-MS/MS) method following an effective overnight enzyme hydrolysis, employing pipette tip solid phase extraction, targeting THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, THCV, and THCVCOOH. Development and validation of the enzymatic hydrolysis and LC-MS/MS method were recently reported [15].

2 Materials

Prepare all solutions with ultrapure water (resistivity of 18 MΩ cm with total organic content <10 ppb) and LCMS-grade solvents.

2.1 Supplies and Equipment

1. β-Glucuronidase from overexpressing recombinant *E. coli* (EBG™; 50,000 U/mL).
2. WAX-S tips (1 mL/20 mg resin with 40 mg salt), DPX Labs, or similar.

3. Volumetric glassware: 5–1000 mL volumetric flasks, 0.5–5 mL class A volumetric pipettes.
4. 12 mL glass screw-top bottles with Teflon-lined screw caps.
5. Adjustable pipettes: 10–100, 100–1000, and 500–5000 µL.
6. 17 × 60 mm glass vials with Teflon-lined screw caps.
7. 1 mL 96-well plates.
8. Plate kit with 500 µL glass inserts and mat cover.
9. 1.5 mL polypropylene microcentrifuge tubes.
10. Magnetic stir plate.
11. pH meter.
12. Shaking water bath.
13. Automated liquid handling system, e.g., Tecan Freedom EVO® 100.
14. Microcentrifuge for 17 × 60 mm tubes and 1.5 mL microcentrifuge tubes.
15. Centrifuge for 96-well plates.
16. Vortexer.
17. LC-MS/MS, e.g., Sciex 5500 QTRAP® triple quadrupole/linear ion trap mass spectrometer with a Turbo V ion spray source coupled with a high-performance liquid chromatography (HPLC) system consisting of a DGU-20A3 degasser, LC-20ADxr pumps, SIL-20ACxr autosampler, and a CTO-20 column oven.
18. Analytical column: UCT Selectra DA 100 × 2.1 mm, 3 µm.
19. In-line filter: Phenomenex KrudKatcher Ultra HPLC.

2.2 Cannabinoid Calibrator and Quality Control Solutions

1. Calibrator intermediate individual analyte stock solutions: 100 mg/L of THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, and THCV. Transfer 0.5 mL of each 1.0 mg/mL stock solution of THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, and THCV to individual 5 mL volumetric flasks. Fill to 5 mL with methanol. Cap, mix well, and transfer to 12 mL glass screw-top bottle.
2. Calibrator 9 10× working standard: 5000 µg/L THCCOOH. Add 0.5 mL of 100 mg/L THCCOOH stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle. Calibrator concentrations are shown in Table 1 (*see* **Note 1**).
3. Calibrator 8 10× working standard: 2500 µg/L of all analytes. Add 250 µL of each 100 mg/L stock solution of THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, THCV, and THCVCOOH into a 10 mL volumetric flask and fill to 10 mL

Table 1
Final concentrations (μg/L) for calibrators in urine

Calibrator	1	2	3	4	5	6	7	8	9
THC	1	2	5	10	20	50	100	250	–
CBG	1	2	5	10	20	50	100	250	–
11-OH-THC	–	2	5	10	20	50	100	250	–
CBD	–	2	5	10	20	50	100	250	–
CBN	–	2	5	10	20	50	100	250	–
THCV	–	2	5	10	20	50	100	250	–
THCVCOOH	–	2	5	10	20	50	100	250	–
THCCOOH	1	2	5	10	20	50	100	250	500

with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.

4. Calibrator 7 10× working standard: 1000 μg/L of all analytes. Add 4 mL of calibrator 8 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
5. Calibrator 6 10× working standard: 500 μg/L of all analytes. Add 5 mL of calibrator 7 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
6. Calibrator 5 10× working standard: 200 μg/L of all analytes. Add 4 mL of calibrator 6 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
7. Calibrator 4 10× working standard: 100 μg/L of all analytes. Add 5 mL of calibrator 5 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
8. Calibrator 3 10× working standard: 50 μg/L of all analytes. Add 5 mL of calibrator 4 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
9. Calibrator 2 10× working standard: 20 μg/L of all analytes. Add 4 mL of calibrator 3 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
10. Calibrator 1 10× working standard: 10 μg/L of all analytes. Add 5 mL of calibrator 2 working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap,

mix well, and transfer to a 12 mL screw-top glass bottle (*see* **Note 2**).

11. Quality control (QC) intermediate individual analyte stock solutions: 100 mg/L of THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, and THCV. Transfer 0.5 mL of each 1.0 mg/mL stock solution of THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, and THCV to individual 5 mL volumetric flasks. Use different 1.0 mg/mL ampules than were used for the calibrators. Fill to 5 mL with methanol. Cap, mix well, and transfer to 12 mL glass screw-top bottle.
12. High QC 10× working standard: 4000 μg/L THCCOOH and 2000 μg/L all other analytes. Add 200 μL of each 100 mg/L stock solution of THC, 11-OH-THC, CBD, CBN, CBG, THCV, and THCVCOOH into a 10 mL volumetric flask. Add 400 μL of 100 mg/L THCCOOH stock solution to the same flask. Fill to volume with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle (*see* **Note 3**).
13. Mid QC 10× working standard: 300 μg/L for all analytes. Add 30 μL of each 100 mg/L stock solution of THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, THCV, and THCVCOOH to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
14. Low QC 10× working standard: 30 μg/L for all analytes. Add 1 mL of mid QC working standard stock solution to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle.
15. Hydrolysis control: 6045 μg/L THCCOOH-glucuronide. Add 604.5 μL of 100 μg/mL THCCOOH-glucuronide standard to a 10 mL volumetric flask and fill to 10 mL with methanol. Cap, mix well, and transfer to a 12 mL screw-top glass bottle. Yields 4000 μg/L THCCOOH if hydrolysis efficiency is 100%.

2.3 Solutions and Buffers

1. Internal standard intermediate stock solution: 10 mg/L of each deuterated compound. Add 0.5 mL of each 100 mg/L stock solution of d_3-THC, d_3-11-OH-THC, d_9-THCCOOH, d_3-CBD, and d_3-CBN to a 5 mL volumetric flask. Fill flask to 5 mL with methanol, cap, and mix well. Store solution in capped, screw-top glass 17 × 60 mm vial at −20 °C.
2. 50 μg/L internal standard working solution: pipette 250 μL of 10 mg/L internal standard intermediate stock solution into a 50 mL volumetric flask. Fill to 50 mL with methanol, cap, and mix well. Transfer working internal standard into a 50 mL

screw-top glass bottle, cap tightly, and store at −20 °C (*see* **Note 4**).

3. 2 M sodium phosphate monobasic solution. Weigh out 60 g of sodium phosphate monobasic, and transfer to a 250 mL volumetric flask. Fill with water to 250 mL, cap, and mix until powder is completely dissolved.
4. 2 M sodium phosphate dibasic solution. Weigh out 71 g of sodium phosphate dibasic and transfer to a 250 mL volumetric flask, fill with water to 250 mL, cap, and mix until powder is completely dissolved.
5. 2 M sodium phosphate buffer, pH 6.8. Transfer 100 mL of 2 M sodium phosphate dibasic solution to a 1 L beaker. Add magnetic stir bar, place on magnetic stir plate at slow stir speed, and insert calibrated pH electrode. While stirring, slowly add 2 M sodium phosphate monobasic solution until pH 6.8 ± 0.1 is achieved. Transfer solution to glass reagent bottle, and store capped at room temperature. Solution expires after 1 month or sooner if precipitants are observed.
6. 5% aqueous formic acid: fill 1000 mL volumetric flask to approximately 50% full with deionized water, add 1000 μL of concentrated formic acid, and fill volumetric flask to 1000 mL with deionized water. Store in screw-top glass reagent bottle at room temperature; stable for 2 weeks.
7. Mobile phase A: 0.15% formic acid in water. Fill a 1000 mL volumetric flask with approximately 500 mL of water, slowly add 1.5 mL of formic acid, fill with water to 1000 mL, cap, and mix well. Transfer solution to 1000 mL glass reagent bottle and place on LC-MS/MS instrument.
8. Mobile phase B: 0.15% formic acid in acetonitrile. Fill a 1000 mL volumetric flask with approximately 500 mL of acetonitrile, slowly add 1.5 mL of formic acid, fill with acetonitrile to 1000 mL, cap, and mix well. Transfer solution to 1000 mL glass reagent bottle and place on LC-MS/MS instrument.

3 Methods

3.1 Sample Preparation

1. Add 200 μL of negative urine to a labeled glass 17 × 60 mm screw-top vial for each calibrator, QC, and hydrolysis control.
2. Add 20 μL of the appropriate calibrator or QC working standard solution to each vial (*see* **Note 5**).
3. Prepare the negative control by pipetting 200 μL of negative urine and 20 μL of methanol into a glass 17 × 60 mm screw-top vial.

4. Pipette 200 µL of each patient urine and 20 µL of methanol into labeled glass 17 × 60 mm screw-top vials.
5. Add 20 µL of working internal standard (containing d_3-THC, d_3-11-OH-THC, d_9-THCCOOH, d_3-CBD, and d_3-CBN) to all calibrators, controls, and patient specimens.
6. Add 50 µL of 2 M sodium phosphate buffer, pH 6.8 to all calibrators, controls, and patient specimens.
7. Add 40 µL of EBG recombinant *E. coli* β-glucuronidase (2000 U/sample) to each vial and mix gently.
8. Cap samples and incubate for 16 h at 37 °C in a shaking water bath.
9. After incubation, transfer samples into 1.5 mL polypropylene microcentrifuge tubes.
10. Add 620 µL of acetonitrile to each tube.
11. Centrifuge at 15,000×*g*, 4 °C for 10 min.
12. Transfer 550 µL of supernatant to a 1 mL, 96-deep-well plate and transfer the plate to an automated liquid handling system.
13. Program the liquid handling system to add 200 µL of 5% aqueous formic acid, and then perform four aspiration/dispense cycles through 1 mL WAX-S tips.
14. Transfer 60 µL from the upper, organic layer into clean 0.5 mL glass inserts residing in a 1 mL 96-well plate containing 140 µL of mobile phase A (*see* **Notes 6** and **7**).
15. Cap inserts, vortex, and centrifuge at 700×*g*, 4 °C for 5 min.
16. Transfer the plate containing inserts to the autosampler.

3.2 Liquid Chromatography Tandem Mass Spectrometry Analysis

1. Analyze specimens on a Sciex 5500 QTRAP with a Selectra DA 100 × 2.1 mm, 3 µm column combined with a KrudKatcher Ultra HPLC in-line filter.
2. Injection volume: 45 µL.
3. Column temperature: 40 °C.
4. Flow rate: 0.5 mL/min.
5. Gradient program (*see* **Note 8**):
   (a) 30% mobile phase B (MPB) for 0.5 min.
   (b) 48% MPB at 1.0 min.
   (c) 75% MPB at 10.0 min.
   (d) 95% MPB at 10.5 min held for 2.0 min.
   (e) Re-equilibrate to 30% MPB over 0.1 min, and hold for 2.0 min.
   (f) Divert flow to waste from 0 to 2.5 min and for the last 6.1 min.
   (g) Total run time of 14.6 min.

6. Mass spectrometer mode: electrospray ionization (ESI) with multiple reaction monitoring (MRM) in positive mode (*see* **Note 9**).
7. Method parameters (*see* **Note 10**):
   (h) Curtain gas flow 45 L/min.
   (i) Medium collision gas.
   (j) Ion spray voltage 5500 V.
   (k) Source temperature 600 °C.
   Ion source gases 1 and 2 were optimized for each MRM period:
   (l) Period I 50 L/min (gas 1) and 60 L/min (gas 2).
   (m) Period II 45 L/min and 70 L/min.
   (n) Period III 50 L/min and 70 L/min.
   (o) Period IV 55 L/min and 70 L/min.
   (p) MS/MS settings are displayed in Table 2.
8. Construct linear calibration curves daily from peak area ratios of analytes to their respective internal standard, with $1/x^2$ weighting.
9. Construct calibration curves from 1 to 250 μg/L for THC and CBG, 2–250 μg/L for 11-OH-THC, CBD, CBN, THCV, and THCVCOOH and 1–500 μg/L for THCCOOH. Figure 1 shows extracted ion chromatograms of representative blank human urine fortified to contain analytes at their low QC concentrations.

4 Notes

1. Store all working calibrator, QC, hydrolysis control, and internal standard solutions at −20 °C. Solutions expire after 6 months.
2. Note that calibrator 1 contains all compounds; however, 11-OH-THC, CBD, CBN, THCV, and THCVCOOH are not quantitated below calibrator 2.
3. QC working solutions at low, medium, and high concentrations are prepared across the linear dynamic range using different ampoules than for calibrators. Store all working QC solutions at −20 °C. Solutions expire after 6 months.
4. Internal standard working and 10 mg/L stock solutions are stored at −20 °C and expire after 6 months.
5. Calibrator, QC, and hydrolysis control 10× methanolic working standard solutions are fortified into urine daily to avoid storage stability issues for cannabinoids in urine [16].

Table 2
LC-MS/MS parameters for cannabinoids, their metabolites, and internal standards in human hydrolyzed urine

Analyte	Q1 (amu)	Q3 (amu)	DP (V)	CE (eV)	CXP (V)	MRM period	Dwell time (msec)
THC	**315.0**	**193.1**	91	31	20	4	58
	315.0	123.1	91	43	14	4	58
11-OH-THC	**331.0**	**201.0**	31	33	18	2	58
	331.0	313.2	31	19	30	2	58
THCCOOH	**345.0**	**193.0**	61	35	14	2	58
	345.0	327.2	61	21	28	2	58
CBD	**315.0**	**193.0**	51	29	18	3	58
	315.0	123.0	51	41	14	3	58
CBN	**311.0**	**223.0**	51	29	18	4	58
	311.0	241.1	51	25	24	4	58
CBG	**317.0**	**193.2**	36	21	14	3	58
	317.0	122.8	36	41	18	3	58
THCV	**287.1**	**165.0**	26	29	14	3	58
	287.1	123.0	26	41	10	3	58
THCVCOOH	**317.0**	**165.1**	31	35	14	1	120
	317.0	271.1	31	25	20	1	120
d_3-THC	**318.1**	**196.1**	76	31	20	4	58
	318.1	123.0	76	43	14	4	58
d_3-11-OH-THC	**334.1**	**201.0**	26	35	16	2	58
	334.1	316.0	26	19	30	2	58
d_9-THCCOOH	**354.1**	**196.1**	56	35	18	2	58
	354.1	335.8	56	23	28	2	58
d_3-CBD	**318.1**	**195.9**	26	27	16	3	58
	318.1	135.2	26	25	10	3	58
d_3-CBN	**314.0**	**223.1**	51	29	18	4	58
	314.0	241.0	51	25	22	4	58

THC delta9-tetrahydrocannabinol, *11-OH-THC* 11-hydroxy-THC, *THCCOOH* 11-nor-9-carboxy-THC, *CBD* cannabidiol, *CBN* cannabinol, *CBG* cannabigerol, *THCV* delta9-tetrahydrocannabivarin, *THCVCOOH* 11-nor-9-carboxy-THCV

Bold masses depicted quantifier transitions. *DP* declustering potential, *CE* collision energy, *CXP* collision cell exit potential, *MRM* scheduled multiple reaction monitoring

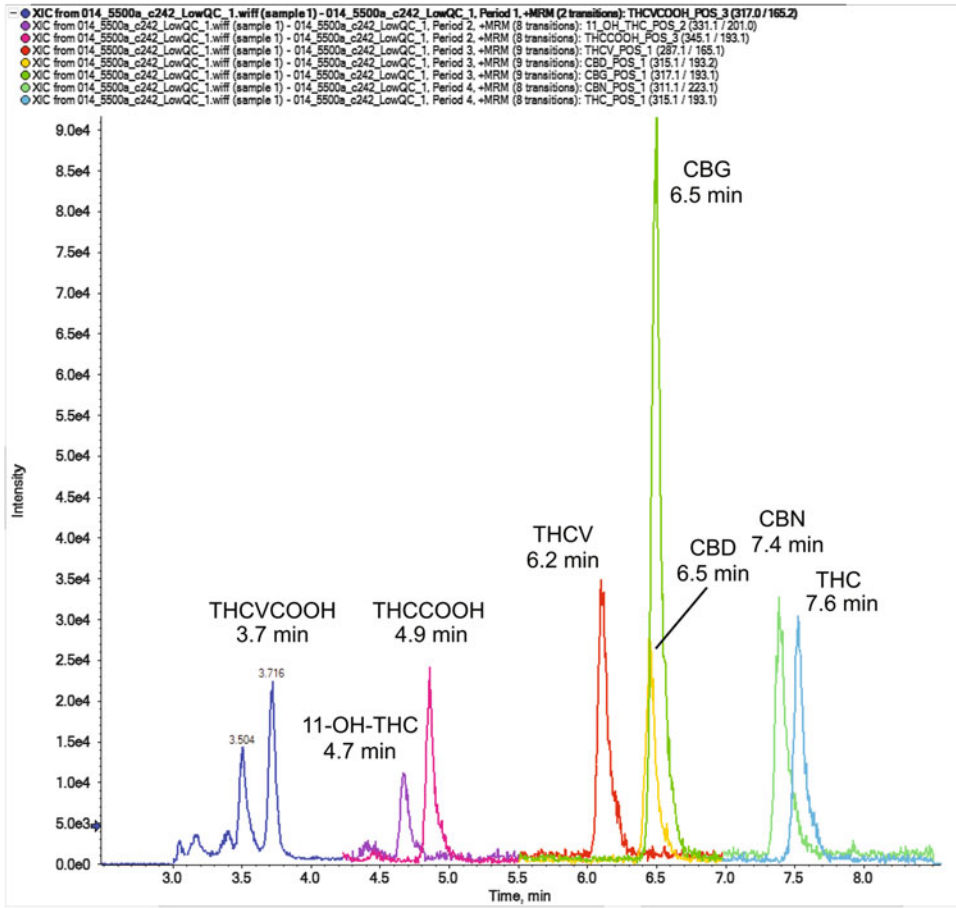

Fig. 1 Extracted ion chromatogram showing quantifier MRM transitions after injecting an extract prepared from 200 μL fortified urine containing each analyte at 3 μg/L. Δ9-Tetrahydrocannabinol (*THC*: *m/z* 315–193), 11-hydroxy-THC (*11-OH-THC*: *m/z* 331–201), 11-nor-9-carboxy-THC (*THCCOOH*: *m/z* 345–193), cannabinol (*CBN*: *m/z* 311–223), cannabigerol (*CBG*: *m/z* 317–193), tetrahydrocannabivarin (*THCV*: *m/z* 287–165), and its metabolite, 11-nor-9-carboxy-THCV (*THCVCOOH*: *m/z* 317–165)

6. The 500 μL glass inserts are used since cannabinoids were found to bind polypropylene plates, while stability was maintained using the glass inserts. More than 50% loss was observed for 2 h. when polypropylene plates (lacking glass inserts) were used in the 4 °C autosampler.
7. Glass inserts are small targets for the automated liquid handling system and autosampler requiring careful adjustment of instrument settings to ensure proper pipetting/injection.
8. If it is desired to target fewer analytes than THC, 11-OH-THC, THCCOOH, CBD, CBN, CBG, THCV, and THCVCOOH, a more simplified (faster) gradient could be implemented; however, full revalidation of the method would be required.

9. Monitor two transitions for each analyte and internal standard. Optimize MS/MS parameters via infusion of individual analytes at 10 μL/min (20 μg/L).
10. Mass spectrometer settings may differ for individual instruments.

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Chapter 3

Analysis of Benzodiazepines for Drug-Facilitated Assaults and Abuse Settings (Urine)

Olaf H. Drummer, Matthew Di Rago, and Dimitri Gerostamoulos

Abstract

An overview of the detection of benzodiazepines and their respective metabolites and target analytes in urine by LC-MS/MS is described. This overview shows substantial differences in the approach to detection using this technique including optional use of β-glucuronidase to hydrolyze conjugates present in urine. There are also significant variations in the extraction method employed from the use of direct injection, liquid-liquid extraction to solid-phase extraction options, with little apparent difference in limits of detection. Chromatography was largely based on the use of C18-bonded columns; however both C8- and phenyl-bonded columns were used to affect separation. Modern-day tandem mass spectrometers are capable of exceptional sensitivity enabling detection of sub-nanogram per milliliter amounts in urine, which provide for longer detection times in the urine of suspected drug-facilitated assaults. A method employed in the laboratory of the authors is provided by way of an example for readers wishing to establish a method in their own laboratory.

Key words Tandem mass spectrometry, Liquid chromatography, Benzodiazepines, Metabolites, β-Glucuronidase, Solid-phase extraction, Liquid-liquid extraction, Novel psychoactive drugs

1 Introduction

Benzodiazepines are a class of drugs that interact with the $GABA_A$ receptor and comprise well over 50 substances that range from short-acting hypnotics to long-acting anxiolytics. Examples of short-acting benzodiazepines include midazolam, oxazepam, and temazepam, while diazepam is a common example of a longer-acting drug used largely to relieve anxiety and act as minor tranquilizers. Increasingly, novel, but illicit, benzodiazepines are becoming available as novel psychoactive drugs that have been detected in many countries [1].

Benzodiazepines are one of the more common drug types to be used by assailants to sedate victims and render them unable or less able to resist an assault (sexual and/or to engage in theft). This crime is often termed drug-facilitated (sexual) assault (DFSA).

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Importantly, most members of this class of drug also cause amnesia, reducing the ability of the victim to remember details of the assault. In a recent review, the incidence of benzodiazepines in victims of DFSA ranged from 3.5% to 38.7% with a median of 11% [2], although they were often in combination with other drugs, including alcohol, illicit, and prescription drugs.

This chapter provides an overview of published techniques for benzodiazepines and their relevant metabolites and close analogs in urine using liquid chromatography tandem mass spectrometry (LC-MS/MS) and provides a method for their determination in urine using this technique.

1.1 Benzodiazepines and Their Analogs

Once consumed benzodiazepines are mostly metabolized and excreted into urine. Metabolites range from simple glucuronide conjugates such as with oxazepam to hydroxylation and subsequent conjugation and/or dealkylation. The dominant form in urine is often a metabolite rather than the parent drug. A few drugs that are structurally unrelated to benzodiazepines bind to a neighboring site of the $GABA_A$ receptor and function largely as hypnotics. These include zolpidem and zopiclone (Z-drugs) and are included in this chapter.

Table 1 lists the individual members of the benzodiazepine class and the related Z-drugs and identifies key metabolites that need to be targeted in a urine analysis. The substances that possess a hydroxy group will usually also exist as a glucuronide metabolite. Hence to achieve maximum sensitivity for detection of the non-conjugated metabolite in urine, a de-conjugation step is advised prior to an extraction or preparation step and introduction into the chromatograph.

1.2 Overview of Published Methods

There have been numerous methods published using LC-MS/MS to determine the presence of benzodiazepines in urine, particularly over the last 10 years. A selection of those specializing in drugs used in assault cases, and in particular benzodiazepines and/or their Z-drug analogs, and relevant metabolite analytes ($n = 20$) are summarized in Table 2 [3–22]. The list is restricted to references published over the last 10 years.

The methods vary substantially in their approach; most (15 of 20) use some form of hydrolysis to liberate conjugated benzodiazepines from their respective glucuronides, although there is significant variation in the source of enzyme used and the hydrolysis conditions. The optimization of these hydrolysis conditions is rarely reported, let alone for all of the known conjugates.

LC-MS/MS used in these publications represented all of the major instrument manufacturers and will vary substantially in sensitivity due to the configuration and design of the mass analyzers. Many of the more recent analyzers show much higher sensitivity than the older LC-MS/MS instruments. Almost all LC-MS/MS

Table 1
Selection of benzodiazepines and related drugs, their key metabolites, and target analytes in urine

Benzodiazepine	Key metabolites	Key analyte for urinalysis
Alprazolam	1-Hydroxy alprazolam	1-Hydroxy alprazolam (hydrolysis)
Bromazepam	3-Hydroxy bromazepam and glucuronides	3-Hydroxy bromazepam (hydrolysis)
Clobazam	Norclobazam (desmethylclobazam)	Clobazam and norclobazam
Clonazepam	7-Acetamido-, 7-amino-, and 3-hydroxy-clonazepam	7-Aminoclonazepam
Clonazolam	7-Amino-, 7-acetamido-clonazolam	7-Aminoclonazolam
Chlordiazepoxide	Demoxepam, oxazepam, and glucuronide	Oxazepam (hydrolysis)
Diazepam	Nordiazepam, oxazepam, temazepam, and their glucuronides	Oxazepam, temazepam (hydrolysis)
Etizolam	1- and 8-hydroxy etizolam	1-Hydroxyetizolam
Estazolam	4-Hydroxy- and 1-oxo-estazolam	4-Hydroxyestazolam (hydrolysis)
Flunitrazepam	7-Acetamido-, 7-aminoflunitrazepam	7-Aminoflunitrazepam
Flurazepam	*N*-1-Desalkyl-, hydroxyethylflurazepam	*N*-1-Desalkylflurazepam (hydrolysis)
Lorazepam	Lorazepam glucuronides	Lorazepam (hydrolysis)
Lormetazepam	Lorazepam and glucuronides	Lorazepam (hydrolysis)
Midazolam	1-Hydroxy midazolam	1-Hydroxy midazolam
Nordiazepam	Temazepam and oxazepam glucuronides	Temazepam and oxazepam (hydrolysis)
Oxazepam	Oxazepam glucuronides	Oxazepam (hydrolysis)
Phenazepam	3-Hydroxy-phenazepam and glucuronides	3-Hydroxy-phenazepam (hydrolysis)
Temazepam	Oxazepam, temazepam, and glucuronides	Oxazepam and temazepam (hydrolysis)
Nitrazepam	7-Acetamido-, 7-aminonitrazepam	7-Aminonitrazepam
Tetrazepam[a]	3-Hydroxyl and glucuronides	Tetrazepam
Triazolam	1-Hydroxy triazolam	1-Hydroxy triazolam (hydrolysis)
Zopiclone	*N*-Desmethyl-zopiclone, zopiclone-*N*-oxide	Zopiclone, *N*-desmethyl-zopiclone
Zolpidem	Carboxy metabolite of 4-methyl	Zolpidem and carboxy metabolite

Note: Hydrolysis refers to hydrolysis of urine liberating conjugated drug or metabolite
[a]Also metabolized to diazepam

Table 2
Analysis parameters for selected publications describing tandem LC-MS detection of benzodiazepines in urine

Reference	Pre-treatment of urine	Extraction	Chromatography conditions	Mass spectrometer, number of benzodiazepine (BZ) analytes, LOD
ElSohly et al. [3]	β-Glucuronidase/sulfatase (4 h, 37 °C)	LLE ChCl3/isopropanol (9:1)	Luna C8 (4.6 × 100 mm, 3 μm), 0.1% acetic acid/ACN	Agilent 1100 SL TOF-MS ESI 22 BZ/metabolites LOD 0.5–3 ng/mL
Glover and Allen [4]	β-Glucuronidase (PV) (0.5 h, 56 °C)	LLE DCM/DCE/heptane/ isopropanol	HyPURITY C8 (3 × 150 mm), 1% HCOOH, 1% isopropanol ACN gradient	API 3000 TQ API 6 BZ LOD 2.5–5 ng/mL
Guale et al. [5]	None	SPE Strata-X-Drug B	Eclipse Plus C18 (3 × 100 mm, 1.8 μm), 5 mM NH_4COO, 5–60% MeOH gradient	Agilent 6230 TOF-MS, 15 BZ/metabolites, LOD not given
Hegsted et al. [22]	β-Glucuronidase (HP-2) (1 h, 65 °C) and without	Direct injection	ACQUITY HSS T3 (2.1 × 100 mm, 1.8 μm), 0.1% HCOOH/MeOH, 50° C	Waters Xevo TQ-S ESI 5 BZ LOD 2–6 ng/mL
Jagerdeo and Schaff [6]	β-Glucuronidase (RA) (0.5 h, 68 °C)	SPE Isolute SLE, DCM/isopropanol (95:5)	Cortex C18 (2.1 × 50 mm, 1.6 μm), 0.1% HCOOH/ACN	Waters Orbitrap 22 BZ/metabolites, LOD 1 ng/mL
Jeong et al. [7]	None	Direct injection	Zorbax SB-C18 (2.1 × 100 mm, 3.5 μm), 2 mM trifluoroacetate/ 0.2% acetic acid ACN gradient	API 3200 TQ ESI 18 BZ LOD 0.3–3 ng/mL
Karampela et al. [8]	None	Direct injection	XTerra MS C8 (2.1 × 250 mm, 5 μm), 0.05% HCOOH/ACN gradient	Shimadzu 2010EV ESI LC-MS, 10 BZ LOD >10 ng/mL

Lee et al. [9]	None	Ethyl acetate LLE	ACE5 C18 (4.6 × 250 mm, 5 μm), 0.1% HCOOH/ACN gradient	API 4000 Q-Trap ESI 21 BZ and others LOD >0.5 ng/mL
Mata et al. [18]	β-Glucuronidase (HP-2) (3 h, 55 °C)	DPX WAX tips	ACQUITY BEH C18 (2.1 × 100 mm, 1.7 μm), water 0.1% HCOOH/ACN 0.1% HCOOH	Waters Xevo TQ-S, ESI 17 BZ, LOD 0.5–12.5 ng/mL
Marin et al. [10]	β-Glucuronidase (BL) (2 h, 60 °C)	SPE Trace-B, ethyl acetate: NH_3 (98:2)	XTerra MS C18 (2.1 × 150 mm, 3.5 μm), 100 mM HCOOH/ ACN gradient	Waters, Micromass, Alliance, Quattro ESI 13 BZ/metabolites LOD <10 ng/mL
Ming and Heathcote [11]	β-Glucuronidase (EC) (1.5 h 60 °C)	Direct injection	ACQUITY BEH C18 (2.1 × 50 mm, 1.7 μm), 0.2% HCOOH/MeOH gradient, 45° C	Waters TQD ESI, 13 BZ/metabolites LOD 0.5–2 ng/mL
Perez et al. [19]	β-Glucuronidase (HP-2) (1 h, 55 °C)	SPE UCT Clean Screen XCEL I, ethyl acetate: NH_4OH (100:2)	ACQUITY BEH C18 (1.7 μm, 2.1 × 50 mm), (A) 0.1% HCOOH and (B) acetonitrile	Waters Quattro micro, ESI 5 BZ, LOD 2–16 ng/mL
Petterson Bergstrand et al. [12]	β-Glucuronidase (10 min, 25 °C)	Direct injection	ACQUITY BEH phenyl column (1.0 × 50 mm, 1.7 μm), ACN/0.1% HCOOH gradient	Waters Xevo TQ ESI 11 designer BZ LOD 1–10 ng/mL
Remane et al. [13]	β-Glucuronidase (EC) (0.5 h, 55 °C) and without	Direct injection	Zorbax Eclipse XDB C18 (4.6 × 100 mm, 5 μm), 50 mM HCOOH	API 4000 Q-Trap ESI 24 BZ/metabolites plus others LOD meet SOFT criteria
Rosano et al. [20]	β-Glucuronidase (1 h, 55 °C)	Direct injection	ACQUITY BEH Phenyl (2.1 × 50 mm, 1.7 μm), 2 mM NH_4COO/2 mM NH_4COO, 0.1% HCOOH in ACN	Waters Xevo TQD, ESI 16 BZ, LOD 25 ng/mL
Salomone et al. [14]	β-Glucuronidase (HP) (1 h, 55 °C)	LLE pH 7.5, DCM/propan-2-ol (85:15)	Eclipse XDB C18 (4.6 × 50 mm, 1.8 μm), MeOH/water gradient	API 3200 triple Q-Trap, 23 BZ/analogs, LOD 0.5–30 ng/mL

(continued)

Table 2
(continued)

Reference	Pre-treatment of urine	Extraction	Chromatography conditions	Mass spectrometer, number of benzodiazepine (BZ) analytes, LOD
Schaefer et al. [15]	β-Glucuronidase (1.5 h, 40 °C)	Direct injection – column switching	Cyclone MAX (2.1 × 50 mm, 5 μm), 10 mM NH_4HCO_3/0.1% HCOOH/ACN	Thermo Fisher TSQ ESI 10 BZ/metabolites LOD mostly <10 ng/mL
Tang et al. [21]	β-Glucuronidase (A) (1 h, 55 °C)	SPE Waters Oasis MCX (combined acidic, basic)	Eclipse C8 (3 × 100 mm, 1.8 μm), 5 mM NH_4COO, 0.1% HCOOH, MeOH	Agilent TQ ESI 14 BZ LOD 2–100 ng/mL
Verplaetse et al. [16]	β-Glucuronidase (PV) (3 h, 65 °C)	Bond Elut Plexa PCX SPE, elution acetone/$CHCl_3$ (1:1), and ammoniated ethyl acetate	ACQUITY C18 (2.1 × 50 mm, 1.7 μm), 10 mM NH_4HCO_3 MeOH gradient	API 3200 Q-Trap, ESI, 29 BZ/metabolites LOD >0.02 ng/mL
Xiong et al. [17]	None	Online SPE (Oasis HLB) with formic acid	XTerra MS C18 (2.1 × 150 mm, 3.5 μm), ACN/MeOH/water	Waters Quattro Premier XE ESI, 8 BZ and glucuronidated metabolites LOD from 0.2 ng/mL

methods listed in Table 2 use electrospray ionization techniques (ESI). However, ESI is more likely to exhibit ion suppression or ion enhancement compared to atmospheric pressure chemical ionization (APCI).

Recently, a series of designer benzodiazepines have appeared throughout much of the world, presumably to overcome laws surrounding availability of benzodiazepines in various countries [1, 23]. These include clonazolam, deschloroetizolam, diclazepam, etizolam, flubromazepam, flubromazolam, flutazolam, meclonazepam, nifoxipam, phenazepam, and pyrazolam [12], although some of these compounds have previously been available in other parts of the world, e.g., phenazepam in Russia. These analytes have been shown to be easily analyzed by LC-MS/MS using a rapid de-conjugation step with β-glucuronidase at 25 °C over 10 min and direct injection of the supernatant into an ultrahigh-pressure liquid chromatograph (UPLC) tandem mass spectrometer under positive ESI. This technique was an adaptation of a previously published method for a wider range of psychoactive drugs in urine that did not utilize a hydrolysis step [24].

One limitation with most methods utilizing LC-MS/MS is when multiple-reaction mode (MRM) is used and preselected transitions are monitored, the resulting method will be limited to identification of known substances, rather than screening for any unknown substances, such as other benzodiazepines. The use of data-dependent acquisition (DDA) modality for ions that exceed a certain threshold is fragmented (and collected) allowing for both screening and identification on a single chromatographic run [25, 26].

The following describes a method used in the authors' laboratory for the detection of a range of psychotropic drugs, including benzodiazepines, in urine. This method is typically used in cases of suspected drug-facilitated assault (*see* **Notes 1**, **2**, and **3**) and generally follows a general unknown screen using one or more methods, such as high-resolution LC-MS, targeted tandem LC-MS, GC-MS, or a benzodiazepine immunoassay screen.

2 Materials

Prepare all buffers in deionized water using chemicals that are at least analytical reagent grade. Use solvents that are of liquid chromatography grade.

Some of the newer substances may be sourced from Toronto Research Chemicals (Canada), Cayman Chemical (USA), or Chiron (Norway).

Deuterated benzodiazepine internal standards are available from AptoChem, Cerilliant, LGC, or Lipomed (*see* **Note 4**).

2.1 Solutions and Reagents (See Note 5)

1. Borate buffer, pH 9.8: Dissolve 0.775 g of boric acid and 4.1 g of sodium tetraborate in 250 mL of water. Adjust to pH 9.8 using 1 M sodium hydroxide.
2. 1.1 M sodium acetate buffer, pH 4.5: Dissolve 22.56 g of anhydrous sodium acetate in 250 mL of water. Adjust to pH 4.5 with glacial acetic acid.
3. 1 M sodium hydroxide (NaOH): Add 4 g of sodium hydroxide to 100 mL of water. Mix well.
4. β-Glucuronidase: Commercial enzyme sourced from red abalone (*Haliotis rufescens*) 7000 units (U) per mL or 14,000 U per sample.
5. Extraction solvent: Dichloromethane/isopropanol/ethyl acetate, 1:1:3 v/v/v. Add 100 mL each of dichloromethane and isopropanol to 300 mL of ethyl acetate, and mix gently.
6. Stock standard: 1 mg/mL each drug or metabolite. Dilute 2.00 mg of each benzodiazepine or metabolite in 2.00 mL methanol. Stock standards can be kept for at least 3 months stored at −20 °C.
7. Working standard 1 (WS1): Dilute 0.1 mL of each stock standard to 1 mL with methanol (*see* **Note 6**).
8. Working standard 2 (WS2): Dilute 0.05 mL of each stock standard to 5 mL with water.
9. Working standard 3 (WS3): Dilute 0.05 mL of each stock standard to 50 mL with water.
10. Mobile phase A: 50 mM ammonium formate pH 3.5. Add 3.15 g of ammonium formate to 1 L of water. Adjust pH to 3.5 with concentrated formic acid.
11. Mobile phase B: 0.1% formic acid in acetonitrile. Add 1 mL of formic acid to 1 L of acetonitrile.

2.2 Supplies and Equipment

1. pH meter.
2. Reciprocating shaker or vortex mixer.
3. Benchtop centrifuge.
4. Nitrogen evaporator.
5. LC-MS/MS such as SCIEX 3200 quadrupole mass spectrometer operated in multiple reaction monitoring modes.
6. Agilent Zorbax Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm particle size) or similar.

3 Methods

3.1 Sample Analysis

1. Obtain drug-free urine (with consent) from a volunteer, and use this to prepare a series of calibration standards for one or more of the benzodiazepine and/or metabolites (*see* **Note 7**). Typically a range of concentrations are prepared at or above the limit of detection to a concentration within the range of the spectrometer and often seen in samples. The typical calibration ranges for some of the more common analytes are listed in Table 3 (*see* **Note 8**).

Table 3
Multiple-reaction mode transitions and typical calibration range for selected benzodiazepines, metabolites, and related analogs

Analyte	Calibration range (mg/L)	Parent mass	First transition	Second transition	Other measured transitions
Alprazolam	0.01–0.25	309	281	205	274
1-Hydroxyalprazolam	0.01–0.25	325	216	189	297, 197
Flunitrazepam	0.005–0.25	314	268	239	183
7-Aminoflunitrazepam	0.005–0.25	284	135	93	226, 227
7-Aminodesmethylflunitrazepam	0.005–0.05	270	121	222	
Hydroxyflunitrazepam	0.005–0.25	330	284	237	
Desmethylflunitrazepam	0.005–0.25	300	254	198	
Clonazepam	0.005–0.25	316	270	214	241
7-Aminoclonazepam	0.005–0.25	286	222	121	250
Nitrazepam	0.0050–0.25	282	236	180	207
7-Aminonitrazepam	0.005–0.25	252	121	208	94, 77
Bromazepam	0.01–0.25	316	182	209	181
Clobazam	0.01–0.25	301	193	224	259, 105, 153
Desmethylclobazam	0.01–0.25	287	245	210	
Chlordiazepoxide	0.01–0.25	300	282	227	
Diazepam	0.01–1	285	154	193	222
Estazolam	0.01–0.25	296	268	206	
Etizolam	0.01–0.25	343	314	259	
Flurazepam	0.01–0.1	388	315	317	134
Desalkylflurazepam	0.01–0.25	289	140	226	
Lorazepam	0.01–0.25	321	275	303	229

(continued)

Table 3
(continued)

Analyte	Calibration range (mg/L)	Parent mass	First transition	Second transition	Other measured transitions
Lormetazepam		337	291	75	289, 317
Midazolam	0.01–0.25	326	291	249	223
Nordiazepam	0.01–1	271	140	165	208
Oxazepam	0.01–1	287	241	269	104
Prazepam	0.005–0.25	325	271	140	272
Phenazepam	0.05–0.50	349	206	179	242
Triazolam	0.005–0.25	343	308	239	
1-Hydroxytriazolam	0.005–0.05	359	331	239	
Temazepam	0.01–1	301	235	177	193, 255, 283
Zopiclone	0.01–0.25	389	245	217	112
Zolpidem	0.01–0.10	308	263	235	236

2. For example, a calibration range of 5–3000 ng/mL is shown below using the prepared working standards (WS1–WS3) for 2 mL urine. These will usually contain several benzodiazepines and/or relevant metabolites.

Concentration (ng per mL of urine)	Volume WS1 (μL)	Volume WS2 (μL)	Volume WS3 (μL)
5			10
10			20
30			60
100		20	
300		60	
1000	20		
3000	60		

3. Obtain at least two control specimens containing a known amount of the benzodiazepine and/or metabolite (*see* **Note 9**).
4. Add 0.5 mL of 1.1 M sodium acetate buffer (pH 4.5) and β-glucuronidase enzyme (14,000 U total) to 2 mL urine speci-

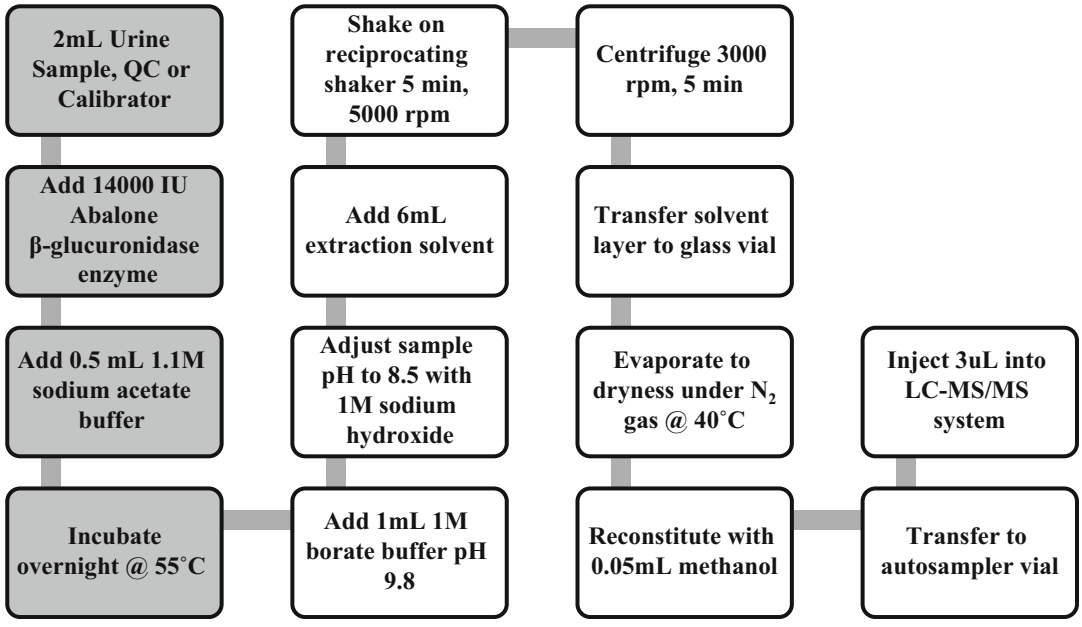

Fig. 1 Flow chart for extraction of benzodiazepines from urine

mens, prepared standards, and control samples. Incubate overnight at 55 °C (*see* **Note 10**).

5. To each hydrolyzed urine sample, add 1 mL of borate buffer (pH 9.8) and 0.3 mL of 1 M sodium hydroxide to adjust pH to 8.5.
6. Add 6 mL of extraction solvent to each sample and gently agitate on a reciprocating shaker or vortex mixer for 5 min (*see* Fig. 1).
7. Centrifuge tubes briefly to separate layers. Separation should occur quickly.
8. Transfer solvent (top layer) to clean glass tubes, and evaporate to dryness under a stream of nitrogen gas at 40 °C.
9. Reconstitute residue in 0.05 mL of methanol and transfer to autosampler vials for analysis.

3.2 Analysis

1. Analytical column: Agilent Zorbax Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm particle size) (*see* **Note 11**).
2. Column temperature: 60 °C.
3. Gradient (can be optimized for separation of similar compounds):

Mobile phase B (%)	Time (min) start	Flow rate (mL/min)
10		1.4
10	1	2.2
100	18	2.2
100	20	2.2
10	20.1	1.4
10	24	1.4

4. Injection volume: 3 μL. Operate the autosampler at room temperature, and rinse the needle with methanol before and after every injection.
5. Analyze by mass spectrometry (*see* **Notes 12** and **13**). Example chromatograms of a standard extract and a DFSA case are shown in Fig. 2.
6. Evaluate MRM for the presence of one of more of the target analytes (*see* **Note 14**). If MRM are present and meet acceptance criteria and substance is above the reporting limit, then the identification can be accepted.
7. An approximate concentration can be calculated based on the calibration responses of the calibrator(s) (*see* **Note 15**).
8. Depending on the type of case and laboratory policy, another test may be required including the presence of identified substance(s) in other specimens (*see* **Notes 16** and **17**).

4 Notes

1. A reference aimed at providing detailed guidance over optimization of LC-MS methods can be found on the web under the Royal Society of Chemistry and the National Measurement System (UK) [27].
2. Analytical methods should be validated to internationally recognized guidelines. An example of validation procedures and performance levels has been published by the Scientific Working Group in Toxicology (SWGTOX, 2013) [28]. Any good-quality HPLC instrument can be used. Window detection threshold of ±5% is usually acceptable for retention time monitoring.
3. The procedure is also useful for the detection of other drugs of interest including drugs of abuse and a range of prescribed and over-the-counter drugs.
4. Deuterated internal standards should be included and paired with the target analytes to correct for any changes in recovery

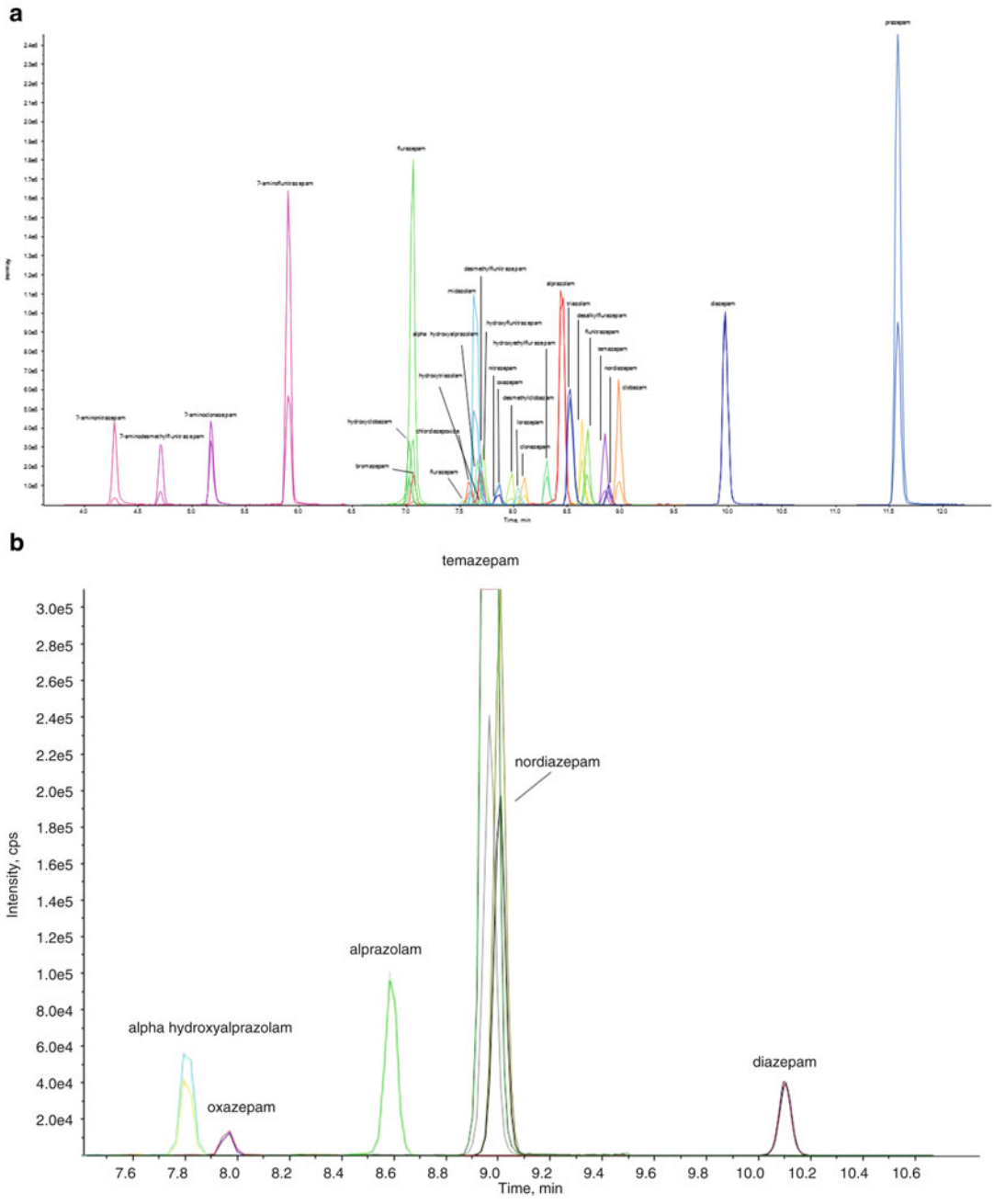

Fig. 2 (**a**) Calibration standard in urine containing benzodiazepines and some metabolites at 0.25 mg/L, except for flurazepam, nordiazepam, and temazepam which are at 0.1 mg/L. (**b**) Chromatogram of a urine from a DFSA case containing alprazolam (0.01 mg/L) and its 1-hydroxy metabolite (0.03 mg/L) and diazepam with three of its metabolites, nordiazepam (0.2 mg/L), oxazepam (0.02 mg/L), and temazepam (0.2 mg/L)

and MS response. A number of these are available for commercial suppliers and include d_5-alprazolam, d_5-diazepam, d_5-nordiazepam, d_5-oxazepam, d_5-temazepam, and the d_5-amino-benzodiazepine metabolites. More than one deuterated internal standard is recommended for longer or more complex runs.

5. Reagents can be stored at ambient temperature for up to 3 months.
6. Calibration standards can be made fresh for each batch or larger batch (e.g., 10 mL) aliquoted into small volumes sufficient for each assay and stored frozen for up to 6 months at −20 °C.
7. When performing a chromatography-based screen prior to this assay, the type of benzodiazepine and its approximate concentration will be suspected. This will give the analyst information on what drugs to include in this method and the corresponding calibration concentrations. It is highly recommended to also include with each batch at least one and preferably several matrix-matched calibrators at various concentrations. These calibrators allow analysts to ensure that both the chromatography and MS conditions are satisfactory and that substances in unknown samples can be confirmed and nominated an approximate concentration. This allows some interpretation to be made with respect to known pharmacokinetics of the drug in question.
8. Samples containing large concentrations may require dilution if a more accurate estimate of concentration is required as the ion source or detector will show saturation effects at higher concentrations, leading to substantial nonlinearity. Due to significant sensitivity of LC-MS/MS techniques, detection limits can easily reach below 1 ng/mL.
9. Quality controls are also highly recommended to include in each batch to provide an indicator of accuracy of quantifications from each analytical run. Commercial controls are available for the more common benzodiazepines. Acceptance limits are generally around 20% of the mean but can be set to two standard deviations obtained from several analytical batches. When dealing with low detection limits, such as often seen in DFSA cases, benzodiazepine/metabolite concentrations may be closer to LOD than at the middle or high end of the calibration range.
10. A hydrolysis marker is recommended, such as oxazepam glucuronide, or any other available glucuronide metabolite to provide a measure of the extent of hydrolysis in each batch. Addition of deuterated glucuronide analyte to each sample can also allow for monitoring of hydrolysis performance in each sample.

11. Many alternative column dimensions, particle sizes, and stationary phases may be used, in which case optimization of instrument conditions is required including flow rate (*see* Table 2).
12. Each LC-MS/MS will require specialized setup and optimization. Parameters such as ion spray voltage; source temperature; curtain, heater, and nebulizing gas flows; detector dwell times, and MRM detection windows will need to be optimized for the compounds to be monitored. Analytes to be detected will require instrument-specific optimization of parent mass and product transitions to be monitored. At least two transitions are required for detection and confirmation, in combination with transition ratio and retention time matching with certified standards. Table 3 summarizes the transitions for most of the more common benzodiazepines and metabolites using the ABI 3200 Q-Trap.
13. Other LC-MS/MS systems can be used, provided that MS parameters have been optimized for all the analytes. Instrument parameters such as source, quadrupole, and detector settings can yield different analyte responses, resulting in varying limits of detection and quantification. These will need to be optimized for each laboratory.
14. Acceptance criteria for transition ratios should be established. This is often $\pm 20\%$ (absolute) of a reference response from a known positive result included in the analytical run; however, criteria need to be established from validation data. If these criteria are not met, then identification has not occurred.
15. Calibration curves are established by calculating peak area ratios for the various calibration standards over the peak area of the respective internal standard and obtaining a regression fit from the lowest to highest calibrator. These regression fits are typically >0.99 (r^2) unless a clear outlier can be determined. The peak area to internal standard ratio for any identified benzodiazepine or metabolite in the specimens is then used in the regression fit to obtain the concentration in the sample. This regression calculation and computation is typically done in software that comes with the instruments, and this may require nonlinear treatment with a quadratic fit most common. Quality controls run with each batch must meet the laboratory acceptance criteria; otherwise an assay would need to be repeated if quantitative data is reported.
16. It is highly recommended that an experienced toxicologist reviews results in case a metabolite can be sourced from ingestion of other benzodiazepines.

17. The Society of Forensic Toxicologists (SOFT) has recommended detection limits and cutoffs for drugs and metabolites of relevance to drug-facilitated assaults; *see* http://www.soft-tox.org/files/MinPerfLimits_DCF2017.pdf.

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Chapter 4

Targeted Opioid Screening Assay for Pain Management Using High-Resolution Mass Spectrometry

Darlington Danso, Loralie J. Langman, and Paul J. Jannetto

Abstract

The use and adherence monitoring of opioids in pain management is recommended by numerous clinical practice guidelines. Many physicians use urine immunoassay screening tests, which suffer from a lack of sensitivity and specificity, to verify compliance to pain medications. However, several immunoassay tests are required to comprehensively detect the synthetic, semisynthetic, and natural opioids due to the limited cross-reactivity of each assay. Superior testing strategies are required to specifically identify low concentrations of opioids found in adherent pain management patients. Therefore we present a method for the qualitative identification of 33 opioids and metabolites (codeine, codeine-6-β-glucuronide, morphine, morphine-6-β-glucuronide, 6-acetylmorphine, hydrocodone, norhydrocodone, dihydrocodeine, hydromorphone, hydromorphone-3-β-glucuronide, oxycodone, noroxycodone, oxymorphone, oxymorphone-3-β-glucuronide, noroxymorphone, meperidine, normeperidine, methadone, EDDP, propoxyphene, norpropoxyphene, tramadol, *O*-desmethyltramadol, tapentadol, tapentadol-β-glucuronide, *N*-desmethyltapentadol, buprenorphine, norbuprenorphine, norbuprenorphine glucuronide, naloxone, naloxone glucuronide, fentanyl, and norfentanyl) in unhydrolyzed urine using a liquid chromatography tandem mass spectrometry (LC-MS/MS) with high-resolution, accurate-mass Orbitrap detection.

Key words Opioids, High-resolution mass spectrometry, Pain management, Accurate-mass

1 Introduction

Opioids are a large class of medications commonly used to relieve acute and chronic pain or help manage opioid abuse and dependence [1, 2]. Medications that fall into this class include buprenorphine, codeine, fentanyl, hydrocodone, hydromorphone, methadone, morphine, oxycodone, oxymorphone, tapentadol, tramadol, and others. Opioids work by binding to the opioid receptors that are found in the brain, spinal cord, gastrointestinal tract, and other organs [3]. Common side effects depend on the dose and include drowsiness, confusion, nausea, constipation, and in severe cases respiratory depression [3]. These medications can also produce physical and psychological dependence and have a high risk

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for abuse and diversion which is one of the main reasons many professional practice guidelines recommend compliance testing in patients prescribed these medications.

Opioids are readily absorbed from the gastrointestinal tract, nasal mucosa, and lungs and after subcutaneous or intramuscular injection. Opioids are primarily excreted from the kidney in both free and conjugated forms. This assay does not hydrolyze the urine sample and therefore allows detection of both parent drugs and metabolites (including glucuronide forms). The detection window for most opioids in urine is approximately 1–3 days with longer detection times for some compounds (e.g., methadone). We describe a liquid chromatography tandem mass spectrometry (LC-MS/MS) method with high-resolution, accurate-mass Orbitrap detection to qualitatively (present vs. not detected) identify 33 opioid compounds (parent drugs and metabolites) in urine to help determine compliance and/or identify illicit opioid drug use. The method is a simple dilute-and-shoot in which urine samples are diluted (1:10) with internal standard (IS) in clinical laboratory reagent water (CLRW) and then subjected to chromatographic separation on a Restek Ultra Biphenyl column and analyzed on the Orbitrap with heated electrospray ionization in positive mode. The mass spectrometry method is full MS followed by data-dependent MS^2. This data-dependent experiment collects full scan data and MS/MS spectra for all compounds defined in the inclusion list. A step gradient elution of the Ultra Biphenyl column achieves a complete chromatographic separation of isobaric compounds with a total run time of 8 min. Compound identification combines exact mass (<5 ppm), delta retention time, and MS^2 spectral library match.

2 Materials

2.1 Samples and Reagents

1. Collect urine samples under proper supervision to ensure specimen integrity and to avoid specimen manipulation that might cause false negative results (*see* **Note 1**).
2. 0.5% formic acid in CLRW. Add 5 mL of formic acid to a 1 L reagent bottle. Bring to volume with CLRW and mix thoroughly. Stable for 1 month at 20–27 °C.
3. Negative (drug-free) urine. Obtain drug-free urine commercially (e.g., UTAK) or from volunteers. Verify as drug-free by assaying prior to use in this method.
4. Wash solution # 1: 2% acetonitrile/1% formic acid in water. Add 20 mL of acetonitrile and 10 mL of formic acid to a 1 L reagent bottle. Bring to volume with CLRW and mix thoroughly. Stable for 1 month at 20–27 °C.

5. Wash solution # 2: 45% acetonitrile/45% isopropanol/10% acetone. Add 450 mL of isopropanol, 450 mL of acetonitrile, and 100 mL of acetone to a 1 L reagent bottle, then mix thoroughly. Stable for 1 month at 20–27 °C.
6. Mobile phase 1: 10 mM ammonium formate/0.1% formic acid in CLRW. Add 1.26 g of ammonium formate, 2 L of CLRW, and 2 mL of formic acid to a 2 L reagent bottle, then mix thoroughly. Stable for 1 month at 20–27 °C.
7. Mobile phase 2: 0.1% formic acid in acetonitrile. Add 2 L of acetonitrile and 2 mL of formic acid to a 2 L reagent bottle, then mix well. Stable for 2 months at 20–27 °C.
8. 50:50 methanol/CLRW. Add 5 mL of methanol and 5 mL of CLRW in a flask and mix well. Make fresh when needed.

2.2 Standards and Controls

Targeted drugs and stock concentrations are shown in Table 1.

1. Intermediate stock internal standards: 10 μg/mL of each deuterated compound. To separate 10 mL volumetric flasks, add 1 mL of each 100 μg/mL stock solution (dihydrocodeine-d6, buprenorphine-d4, norbuprenorphine-d3, fentanyl-d5, norfentanyl-d5) or 100 μL of each 1 mg/mL stock solution (oxycodone-d6 or methadone-d9). Bring to volume with methanol. Stable ≤−10 °C in amber glass vials with Teflon caps for 2 years.
2. Working internal standard: 7.5 ng/mL of oxycodone-d6, dihydrocodeine-d6, and methadone-d9, 1.0 ng/mL of fentanyl-d5 and norfentanyl-d5, and 2.5 ng/mL of buprenorphine-d4 and norbuprenorphine-d5. Add 75 μL of the oxycodone-d6 intermediate, 75 μL of the dihydrocodeine-d6 intermediate, 75 μL of the methadone-d9 intermediate, 25 μL of the buprenoprhine-d4 intermediate, 25 μL of the norbuprenorphine-d5 intermediate, 10 μL of the fentanyl-d5 intermediate, and 10 μL of the norfentanyl-d4 intermediate stock solution to a 100 mL volumetric flask. Bring to volume with 0.5% formic acid in CLRW. Mix well for 20–30 min. Stable for 2 months at 2–8 °C.
3. Intermediate opioid standard: 10 μg/mL of each drug. Add 100 μL of each 1.0 mg/mL stock of methadone, EDDP, meperidine, cis-tramadol, tapentadol, o-desmethyltramadol, dihydrocodeine, n-desmethyltramadol, norhydrocodone, oxycodone, noroxycodone, hydromorphone, oxymorphone, normeperidine, morphine, noroxymorphone, hydrocodone, codeine, and naloxone to a 10 mL volumetric flask. Bring to volume with methanol, and mix well. Stable at or below −10 °C in amber glass vials with Teflon caps for 2 years or until manufacturer expiration of stock I, whichever comes first.

Table 1
Standard concentrations (stock and intermediate calibration solution), negative control, cutoff, and positive control values for the targeted opioid screen

Drug	Stock concentration (mg/mL)	Intermediate concentration (μg/mL)	Negative control value (ng/mL)	Cutoff value (ng/mL)	Positive control value (ng/mL)
Codeine	1.0	10.0	10.0	25.0	50.0
Codeine-6-β-glucuronide	0.1	10.0	50.0	100.0	200.0
Morphine	1.0	10.0	10.0	25.0	50.0
Morphine-6-β-glucuronide	1.0	10.0	50.0	100.0	200.0
6-Acetylmorphine	1.0	10.0	10.0	25.0	50.0
Hydrocodone	1.0	10.0	10.0	25.0	50.0
Norhydrocodone	1.0	10.0	10.0	25.0	50.0
Dihydrocodeine	1.0	10.0	10.0	25.0	50.0
Hydromorphone	1.0	10.0	10.0	25.0	50.0
Hydromorphone-3-β-glucuronide	0.1	10.0	50.0	100.0	200.0
Oxycodone	1.0	10.0	10.0	25.0	50.0
Noroxycodone	1.0	10.0	10.0	25.0	50.0
Oxymorphone	1.0	10.0	10.0	25.0	50.0
Oxymorphone-3-β-glucuronide	0.1	10.0	50.0	100.0	100.0
Noroxymorphone	1.0	10.0	10.0	25.0	50.0
Fentanyl	1.0	1.0	1.0	2.0	5.0
Norfentanyl	1.0	1.0	1.0	2.0	5.0
Meperidine	1.0	10.0	10.0	25.0	50.0
Normeperidine	1.0	10.0	10.0	25.0	50.0
Naloxone	1.0	10.0	10.0	25.0	50.0
Naloxone-3-β-glucuronide	1.0	10.0	50.0	100.0	200.0
Methadone	1.0	10.0	10.0	25.0	50.0
EDDP	1.0	10.0	10.0	25.0	50.0
Propoxyphene	1.0	10.0	10.0	25.0	50.0
Norpropoxyphene	1.0	10.0	10.0	25.0	50.0
Tramadol	1.0	10.0	10.0	25.0	50.0

(continued)

Table 1
(continued)

Drug	Stock concentration (mg/mL)	Intermediate concentration (μg/mL)	Negative control value (ng/mL)	Cutoff value (ng/mL)	Positive control value (ng/mL)
O-Desmethyltramadol	1.0	10.0	10.0	25.0	50.0
Tapentadol	1.0	10.0	10.0	25.0	50.0
N-Desmethyltapentadol	1.0	20.0	20.0	50.0	100.0
Tapentadol-β-glucuronide	0.1	10.0	50.0	100.0	200.0
Buprenorphine	1.0	2.0	2.0	5.0	10.0
Norbuprenorphine	1.0	2.0	2.0	5.0	10.0
Norbuprenorphine glucuronide	1.0	10.0	10.0	20.0	50.0

4. Intermediate glucuronide standard: 10 μg/mL of each conjugated metabolite. Add 1.0 mL of each 100 μg/mL stock of morphine-6β-D-glucuronide, naloxone-3β-D-glucuronide, tapentadol-β-D-glucuronide, codeine-6β-D-glucuronide, oxymorphone-3β-D-glucuronide, and hydromorphone-3β-D-glucuronide to a 10 mL volumetric flask. Bring to volume with 50:50 methanol/CLRW and mix well. Stable at or below −10 °C in amber glass vials with Teflon caps for 2 years or until manufacturer expiration of stock I, whichever comes first.
5. 10 μg/mL norbuprenorphine glucuronide intermediate standard (*see* **Note 2**). Add 100 μL of the 1.0 mg/mL norbuprenorphine glucuronide stock to a 10 mL volumetric flask. Bring to volume with methanol, and mix well. Stable at or below −10 °C in amber glass vials with Teflon caps for 2 years or until manufacturer expiration of stock I, whichever comes first.
6. 1.0 μg/mL fentanyl and norfentanyl intermediate standard. Add 10 μL of each 1.0 mg/mL stock of fentanyl and norfentanyl to a 10 mL volumetric flask and bring to volume with methanol. Stable at or below −10 °C in amber glass vials with Teflon caps for 2 years or until manufacturer expiration of stock I, whichever comes first.
7. 2.0 μg/mL buprenorphine and norbuprenorphine intermediate standard. Add 20 μL of each 1.0 mg/mL stock of buprenorphine and norbuprenorphine to a 10 mL volumetric flask and bring to volume with methanol. Stable at or below −10 °C in amber glass vials with Teflon caps for 2 years or until manufacturer expiration of stock I, whichever comes.

8. 20 μg/mL *N*-desmethyltapentadol intermediate standard. Add 200 μL of 1.0 mg/mL *N*-desmethyltapentadol stock to a 10 mL volumetric flask and bring to volume with methanol. Stable at or below −10 °C in amber glass vials with Teflon caps for 2 years or until manufacturer expiration of stock I, whichever comes.
9. Cutoff calibrator. To a 50 mL volumetric flask, add 100 μL of the fentanyl and norfentanyl intermediate. Add 125 μL of the buprenorphine and norbuprenorphine intermediate. Add 125 μL of the *N*-desmethyltapentadol intermediate. Add 100 μL of the norbuprenorphine glucuronide intermediate. Add 500 μL of the intermediate glucuronide standard. Add 125 μL of the intermediate opioid standard. Bring to volume with negative urine, then mix well for 30 min. Stable for 1 year at −10° to −35 °C.
10. Negative control: ≤50% of the cutoff concentration. To a 50 mL volumetric flask, add 50 μL of the fentanyl and norfentanyl intermediate. Add 50 μL of the buprenorphine and norbuprenorphine intermediate. Add 50 μL of the *N*-desmethyltapentadol intermediate. Add 50 μL of the norbuprenorphine glucuronide intermediate. Add 250 μL of the intermediate glucuronide standard. Add 50 μL of the intermediate opioid standard. Bring to volume with negative urine, and then mix well for 30 min. Stable for 1 year at −10° to −35 °C.
11. Positive control: ≥200% of the cutoff concentration. To a 50 mL volumetric flask, add 250 μL of the fentanyl and norfentanyl intermediate. Add 250 μL of the buprenorphine and norbuprenorphine intermediate. Add 250 μL of the *N*-desmethyltapentadol intermediate. Add 250 μL of the norbuprenorphine glucuronide intermediate. Add 1000 μL of the intermediate glucuronide standard. Add 250 μL of the intermediate opioid standard. Bring to volume with negative urine, and then mix well for 30 min. Stable for 1 year at −10° to −35 °C.

2.3 Supplies and Equipment

1. 12 × 75 mm disposable borosilicate glass test tubes (manual method/daily prime).
2. Microplate 96 deep-well PP square well, 2 mL/well, DNase/RNase free.
3. Restek Ultra BiPh analytical column, 50 × 3.0 mm, 3 μm.
4. Centrifuge with rotors for 12 × 75 mm test tubes.
5. Adhesive seal.
6. Vortexer.
7. TraceFinder 4.1 clinical research software.

8. Thermo Scientific Q Exactive Plus, Dionex UltiMate degasser 3000, Leap Ctc Pal auto-injector, or equivalent, and Dionex UltiMate 3000 RS Pump.

3 Methods

3.1 Extraction Procedure (see Note 3)

1. Mix all patient samples briefly, aliquot into labelled 12 × 75 mm disposable borosilicate glass test tubes, and centrifuge for 10 min at 2850 g.
2. Aliquot 100 µL of each blank, standard, control, and patient sample into its own sample well of a 96-well plate. This assay uses a one point calibration (the cutoff calibrator), a negative control, a positive control, and a blank (drug-free urine) with each batch (*see* **Note 4**).
3. Add 900 µL of working internal standard to each well, and then mix by vortexing.
4. Cover the plate with an adhesive seal for analysis.

3.2 Analysis

1. Injection volume: 20 µL.
2. Flow rate: 0.50 mL/min.
3. Total run time: 8 min.
4. Mobile-phase gradient conditions are shown in Table 2 (*see* **Notes 5** and **6**). Figure 1 shows chromatograms demonstrating separation of isobaric compounds.
5. Mass spectrometer source conditions are shown in Table 3.
6. Analyze data: Compound identification is by retention time, exact masses (m/z) at 5 ppm (Table 4), and spectral library match (*see* **Note 7**).

Table 2
Gradient conditions

Start	Time (s)	% Mobile phase B
0.00	30	0
0.50	250	20
4.67	50	100
5.50	35	100
6.08	130	0

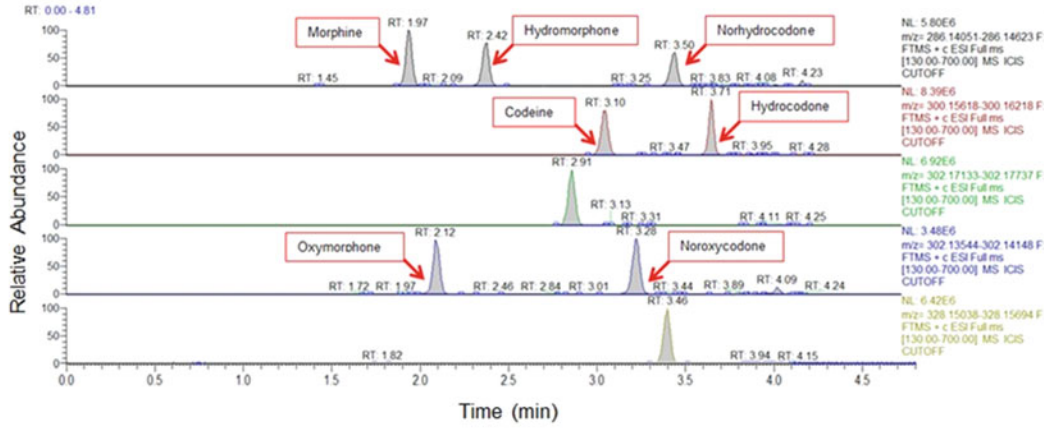

Fig. 1 Chromatograms showing the separation of isobaric compounds

Table 3
Mass spec parameters (source conditions)

HESI source	Actual
Sheath gas flow rate	55
Aux gas flow rate	15
Sweep gas flow rate	2
Spray voltage (kv)	3.50
Spray current (μA)	0.0
Capillary temp (°C)	320
S-lens RF level	
Aux gas heater temp (°C)	350

4 Notes

1. Common adulterants known to affect results are water, soap, bleach, vinegar, and salt. Preferred specimen volume size is 5 mL and a minimum of 1.0 mL. From the results of 28 days' stability studies conducted, samples stored refrigerated were stable for 14 days, those stored frozen were stable for 28 days, and those stored ambient were stable for 72 h. Icteric and hemolyzed samples are rejected.
2. With a lower cutoff value (10 μg/mL) than the rest of the glucuronides, the norbuprenorphine glucuronide intermediate standard is made by itself. Unique intermediates of fentanyl, norfentanyl, buprenorphine, norbuprenorphine, and

Table 4
Transitions and retention times for drug identification

Drug	*m/z*	Rt (min)	Drug	*m/z*	Rt (min)
Codeine	300.15918	3.10	Meperidine	248.16428	3.86
Codeine-6-β-glucuronide	476.19151	2.62	Normeperidine	234.14871	3.85
Morphine	286.14337	1.97	Naloxone	328.15433	2.90
Morphine-6-β-glucuronide	462.17586	1.77	Naloxone-3-β-glucuronide	504.18642	1.82
6-Acetylmorphine	328.15359	3.37	Methadone	310.21628	4.10
Hydrocodone	300.15912	3.71	EDDP	278.19012	4.11
Norhydrocodone	286.14328	3.50	Propoxyphene	340.22687	4.02
Dihydrocodeine	302.17435	2.83	Norpropoxyphene	308.20056	4.11
Hydromorphone	286.14346	2.42	Tramadol	264.19547	3.83
Hydromorphone-3-β-glucuronide	462.17586	1.43	*O*-Desmethyltramadol	250.17993	3.12
Oxycodone	316.15387	3.48	Tapentadol	222.18501	3.83
Noroxycodone	302.13846	3.28	*N*-desmethyltapentadol	208.16959	3.83
Oxymorphone	302.13837	2.12	Tapentadol-β-glucuronide	398.21713	3.29
Oxymorphone-3-β-glucuronide	478.17080	1.08	Buprenorphine	468.31061	3.97
Noroxymorphone	288.12303	1.63	Norbuprenorphine	414.26343	3.85
Fentanyl	337.22711	3.97	Norbuprenorphine glucuronide	590.29597	3.51
Norfentanyl	233.16461	3.76			

Rt retention time

n-desmethyltapentadol are made for similar reasons. This makes the preparation of the working standard and controls in the matrix easier.

3. All procedures are carried out at room temperature. Standard, controls, and reagents are allowed to equilibrate to room temperature before using. All necessary precautions should be taken when it comes to handling of samples to avoid potential infections.
4. Sample preparation could be readily automated. Carryover studies should always be investigated during method development to rule out the possibility of false positives from spiked patient samples and also from analytes with high concentration.
5. Interference studies for the common prescribed drugs, over-the-counter drugs, therapeutic drugs, and common drugs of

abuse should be conducted during method development. Matrix effects should also be investigated during method development.

6. The gradient elution shown off of Ultra Biphenyl Restek column achieved complete chromatographic separation of isobaric compounds.
7. Since this is a qualitative assay, results are reported "present" when analyte concentrations are above the cutoff or "not detected" when concentrations are below the cutoff. Acceptance criteria are expected retention time and actual retention time of each analyte within ±0.10 min, better than 5 ppm mass accuracy; and spectral library score >80%.

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Chapter 5

Measurement of Buprenorphine and Norbuprenorphine in Urine

Andrea R. Terrell, Vipin Adhlakha, and Poluru Reddy

Abstract

Buprenorphine, a synthetic opioid possessing both analgesic and opioid receptor antagonist properties, has proven to be an effective therapeutic aid for opioid dependency and chronic pain management. The downside, as with all opioids, natural or synthetic, is its potential for misuse and abuse. The euphoria induced by buprenorphine leads to abuse. Additionally, individuals with an active addiction to short-acting opioids such as heroin may use buprenorphine between doses of their drug of choice to stave off withdrawal symptoms. As such, buprenorphine monitoring is utilized in medication-assisted therapy programs for opioid dependency, as well as chronic pain management settings. Buprenorphine may also be included in drug testing programs for law enforcement purposes. The assay described here was designed to detect and quantify both buprenorphine and its metabolite norbuprenorphine.

Key words Buprenorphine, Norbuprenorphine, LC-MS/MS, Opioid

1 Introduction

Buprenorphine is a versatile synthetic opioid. In the 1970s buprenorphine was used as a low-dose analgesic for postoperative and cancer patients. Reports of abuse began soon after its introduction. In 1985 the United States classified buprenorphine as a schedule V narcotic analgesic [1]. In 2002 the DEA placed all formulations containing buprenorphine onto schedule III and approved it for medication-assisted treatment of opioid dependency [2].

According to statistics provided by the Drug Abuse Warning Network (DAWN), buprenorphine-related ED visits involving nonmedical use did not reach a measurable level until 2006, when there were a reported 4440 visits to emergency departments. The number of these visits then increased 384% to 21,483 visits in 2011. More recently, visits increased 51% from 14,266 visits in 2009 to 21,483 visits in 2011 [3].

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It has 25–40 times the analgesic efficacy of morphine [4]. The primary receptors that mediate opioid effects are the μ and the κ receptors. Buprenorphine's versatility lies in its dual mechanisms of action, having both μ (agonist) and κ (antagonist) mediating properties. This facilitates the drug's effectiveness in opioid dependency treatment (antagonist activity) and in chronic pain management (agonist activity). The drug may also be slowly withdrawn without the discomfort that often accompanies methadone withdrawal.

Buprenorphine's bioavailability is maximized with sublingual administration. Sublingual formulations of buprenorphine, alone or in combination with naloxone, are available. When used as directed, the buprenorphine/naloxone combination delivers the analgesic and antagonist effects of the buprenorphine, without the full antagonist effects of the naloxone. If the combination drug is injected, the bioavailability of the naloxone is increased which precipitates withdrawal and blocks the euphoric and analgesic properties in opioid-dependent individuals [5]. An extended release transdermal formulation designed for relief of moderate to severe chronic pain was approved in 2010. In a study comparing sublingual vs. transdermal buprenorphine in osteoarthritis patients, both forms were similarly effective, with transdermal buprenorphine demonstrating better tolerability.

Buprenorphine is metabolized in the liver via the CYP3A4 pathway to norbuprenorphine, its primary metabolite. Both buprenorphine and norbuprenorphine are pharmacologically active. Accordingly, both compounds should be monitored. Additionally, both parent and metabolite are extensively metabolized to their glucuronide conjugates. Drug monitoring in urine is performed for purposes of assessing compliance or detecting illicit use; thus, it is desirable to measure total drug in the sample. Free buprenorphine and norbuprenorphine generally represent just 1% of a dose, so hydrolysis is performed prior to measurement in urine.

Commercially available immunoassays are often used to screen for buprenorphine in urine samples. Liquid chromatography with mass spectrometric detection is now commonly used for confirmation, or as the presumptive and definitive test, if immunoassay is not used. Enzymatic hydrolysis has proven to be more effective than acid or base hydrolysis for releasing the glucuronide conjugate from buprenorphine and norbuprenorphine.

This chapter describes a method for quantitative determination of total buprenorphine and norbuprenorphine in urine utilizing liquid chromatography with tandem mass spectrometry (LC-MS/MS). The method, as described here, was extracted from a larger analytical procedure for the quantitative analysis of 48 drugs and metabolites in urine. The chromatography conditions described in this chapter have been optimized for the 48-drug panel. Adjustments should be made to optimize for a method that contains only buprenorphine, norbuprenorphine, and corresponding internal standards.

2 Materials

2.1 Prepared Reagents (See Note 1)

1. **Mobile phase A**: 0.1% formic acid in water: Break 1 ampule of high-grade formic acid and transfer the entire contents (1 mL) using a glass pipette (*see* **Note 2**) into 1 L of HPLC grade water. Store at room temperature for up to 1 month (*see* **Note 3**).
2. **Mobile phase B**: 0.1% formic acid in methanol: Break 1 ampule of high-grade formic acid and transfer the entire contents (1 mL) using a glass pipette into 1 L of HPLC grade methanol. Store at room temperature for up to 1 month.
3. **Needle rinse**: 60/20/20 isopropanol/methanol/acetonitrile: Combine 200 mL of methanol, 200 mL of acetonitrile, and 600 mL of isopropanol. Store at room temperature for up to 1 month.
4. **200 mM phosphate buffer, pH 6.8:** Weigh out 8.0 g of sodium phosphate monobasic dihydrate and 8.7 g of sodium phosphate dibasic dihydrate and mix to dissolve in 500 mL of water. Using pH paper or a pH meter, verify pH is 6.8 (±0.2). Store at room temperature for up to 2 months.
5. **20 mM ammonium formate buffer in 80/20 water/methanol, pH 3.7**: Weigh 250 mg of ammonium formate and dissolve in 160 mL of mobile phase A. Add 40 mL of mobile phase B. Using pH paper or a pH meter, verify pH is 3.7 (±0.2). Store at room temperature for up to 1 month.
6. **Internal Standard (IS) spiking solution: 50 ng/mL each IS.** Combine 10 µL of 100 µg/mL buprenorphine-d_4 stock solution, 10 µL of 100 µg/mL norbuprenorphine-d_3 stock solution, 5 mL of acetonitrile, and 15 mL of water in a glass or polypropylene tube. Mix well. Store refrigerated for up to 1 month.
7. **Composite mix solution (CMS BUP): 10,000 ng/mL buprenorphine and norbuprenorphine.** Combine 100 µL of 100 µg/mL buprenorphine standard, 100 µL of 100 µg/mL norbuprenorphine standard, and 800 µL of acetonitrile in a glass screw cap vial. Mix well.
8. **Composite spiking solution (CSS) 1: 2000 ng/mL buprenorphine and norbuprenorphine.** Combine 200 µL of CMS BUP with 800 µL of acetonitrile in a glass screw cap vial. Store at −20 °C for up to 2 months.
9. **CSS 2: 400 ng/mL buprenorphine and norbuprenorphine.** Mix 200 µL of CSS 1 with 800 µL of acetonitrile in a glass screw cap vial. Store at −20 °C for up to 2 months.

10. **CSS 3: 100 ng/mL buprenorphine and norbuprenorphine.** Mix 200 µL of CSS 2 with 600 µL of acetonitrile in a glass screw cap vial. Store at −20 °C for up to 2 months.
11. **CSS 4: 20 ng/mL buprenorphine and norbuprenorphine.** Mix 100 µL of CSS 3 with 400 µL of acetonitrile in a glass screw cap vial. Store at −20 °C for up to 2 months.
12. **System suitability solution:** Combine 4 mL of water and 1 mL of methanol in a glass or polypropylene vial. Add 10 µL of CSS 1, mix, and store refrigerated for up to 1 month.
13. **Quality control (QC) mix solution (QMS BUP):** Using a different aliquot of stock standard material as the calibrators, combine 100 µL of 100 µg/mL buprenorphine standard, 100 µL of 100 µg/mL norbuprenorphine standard, and 800 µL of acetonitrile in a glass screw cap vial. Mix well. Store at −20 °C for up to 2 months.
14. **QC composite spiking solution 1 (QCC 1): 2000 ng/mL buprenorphine and norbuprenorphine.** Combine 200 µL of QMS BUP with 800 µL of acetonitrile in a glass screw cap vial. Mix well. Store at −20 °C for up to 2 months.
15. **QCC 2: 500 ng/mL buprenorphine and norbuprenorphine.** Combine 100 µL of QCC1 with 300 µL of acetonitrile in a glass screw cap vial. Mix well. Store at −20 °C for up to 2 months.

2.2 Preparation of Urine Calibrators and Controls

1. Cal 1: 2 ng/mL both analytes. Combine 100 µL of CSS 4 and 900 µL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months (*see* **Note 4**).
2. Cal 2: 5 ng/mL both analytes. Combine 50 µL of CSS 3 and 950 µL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months.
3. Cal 3: 10 ng/mL both analytes. Combine 100 µL of CSS 3 and 900 µL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months.
4. Cal 4: 20 ng/mL both analytes. Combine 50 µL of CSS 2 and 950 µL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months.
5. Cal 5: 50 ng/mL both analytes. Combine 125 µL of CSS 2 and 875 µL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months.
6. Cal 6: 100 ng/mL both analytes. Combine 50 µL of CSS 1 and 950 µL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months.

7. Cal 7: 200 ng/mL both analytes. Combine 100 μL of CSS 1 and 900 μL of urine in a glass screw cap vial. Store at −20 °C for up to 2 months.
8. QC: low – 6 ng/mL both analytes. Add 4.6 mL of blank urine to tube, followed by 340 μL of acetonitrile, and then 60 μL of QCC 2. Vortex well. Store at −20 °C for up to 2 months.
9. QC: mid – 18 ng/mL both analytes. Add 4.6 mL of blank urine to tube, followed by 355 μL of acetonitrile, and then 45 μL of QCC 1. Vortex well. Store at −20 °C for up to 2 months.
10. QC: high – 72 ng/mL both analytes. Add 4.6 mL of blank urine to tube, followed by 220 μL of acetonitrile, and then 180 μL of QCC 1. Vortex well. Store at −20 °C for up to 2 months.

2.3 Supplies and Analytical Equipment

1. Sciex 4500 system with Turbo V Source and diverter valve.
2. MultiQuant software v3.0 or later.
3. Shimadzu Nexera XR HPLC System.
4. LC-20AD XR binary pumps.
5. SIL-20AC Autosampler.
6. CTO-20AC column oven.
7. CBM-20A System Controller.
7. Phenomenex HPLC column, 2.6 μm, Kinetex Biphenyl, 50 × 3.0 mm.
8. Phenomenex SecurityGuard ULTRA UHPLC Biphenyl Cartridges, 3.0 mm.
9. Phenomenex SecurityGuard ULTRA Holder.
10. Screw cap glass vials capable of holding 2 mL.
11. Glass autosampler vials with inserts.
12. β-glucuronidase (IMCSzyme [recombinant], >50,000 U/mL, or similar).
13. 1.5 mL or 2.0 mL microcentrifuge tubes.
14. Polypropylene tubes capable of holding >5 mL.
15. pH meter or pH paper.

3 Method

3.1 Sample Preparation

1. Aliquot 50 μL of urine from each calibrator, QC, and sample into a microcentrifuge tube.
2. For each double blank and negative control, aliquot 50 μL of blank urine.

3. Add 50 µL of the composite IS spiking solution to each of the calibrators, QCs, negative controls, and samples and then mix. Do not add IS to the double blanks.
4. Add 50 µL of 200 mM phosphate buffer pH 6.8 and vortex.
5. Add 20 µL IMCS enzyme (50,000 U/mL) and mix gently.
6. Incubate at 55 °C for 30 min.
7. Add 200 µL of 20 mM ammonium formate buffer pH 3.7 and vortex.
8. Centrifuge at 30,000×*g* for 10 min.
9. Transfer approximately 200 µL of supernatant to autosampler vials with inserts and cap. Store refrigerated until analysis.

3.2 Analysis

1. Inject 100 µL of the system suitability standard before each batch.
2. Injection volume: 5 µL.
3. Needle rinse mode: before and after aspiration.
4. Autosampler temperature: 8 °C.
5. Flow rate: 0.7 mL/min.
6. Column oven temperature: 40 °C.
7. Gradient conditions are shown in Table 1.
8. Divert flow to waste for the first 1 min.
9. Mass spectrometer conditions are shown in Table 2.
10. Analyte-specific parameters are shown in Table 3.
11. Process the data using the first transition for each analyte as the quantifier, and the second transition as the ion ratio qualifier.
12. Use linear regression model with $1/x^2$ weighting for all analytes.

Table 1
Chromatography gradient

Time (min)	% Mobile phase B
1.5	20
3.0	42
4.6	82
4.7	95
5.2	95
5.21	5
6.0	(Stop)

Table 2
Mass spectrometer parameters

Parameter	Setting
Polarity	ESI positive/negative switching
Curtain gas	30 psi
CAD gas	10
Ionspray voltage (Pos)	2500 V
Ionspray voltage (Neg)	−4500 V
Temperature	550 °C
Ion source gas 1	60 psi
Ion source gas 2	60 psi
Acquisition time	5.9 min
Q1 resolution	Unit
Q3 resolution	Unit
MRM pause time	3 ms
Settling time	50 ms
MRM window (Pos)	40 s
Target scan time (Pos)	0.3 s
MRM window (Neg)	20 s
Target scan time (Neg)	0.05 s
EP	10 V

Table 3
Analyte-specific parameters

Q1 mass	Q3 mass	RT (min)	Analyte	DP	CE	CXP
468.2	414.2	4.66	Buprenorphine 1	100	50	16
468.2	396.1	4.66	Buprenorphine 2	100	54	14
468.2	55.1	4.66	Buprenorphine 3	100	92	14
472.2	59.1	4.66	Buprenorphine-d_4	100	92	14
414.2	152.0	4.34	Norbuprenorphine 1	120	130	12
414.2	115.0	4.34	Norbuprenorphine 2	120	124	8
414.2	165.0	4.34	Norbuprenorphine 3	120	98	12
417.2	83.1	4.34	Norbuprenorphine-d_3	120	74	8

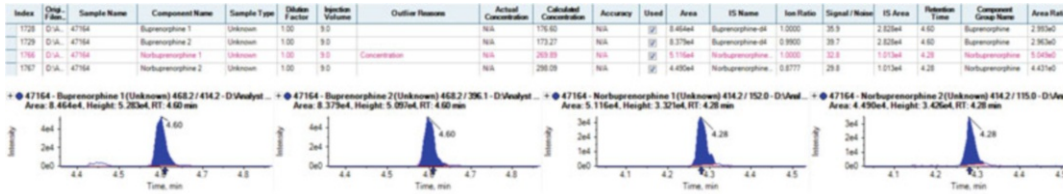

Fig. 1 Positive sample from patient using Suboxone

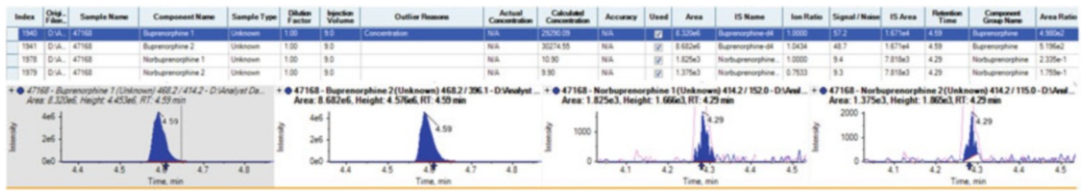

Fig. 2 A sample that has been adulterated by "pill scraping" where a small amount of pill or film is directly added to the urine sample. In this scenario, samples will have an elevated concentration of parent drug and little to no metabolite

Fig. 3 Negative sample

13. Confirm that batch meets acceptance criteria (*see* **Note 5**).
14. Report patient samples using a cutoff of 10 ng/mL. Results below this are reported as negative or not detected (*see* **Note 6**). Figures 1, 2, and 3 show examples of patient results using this method.

4 Notes

1. All reagents should be HPLC grade or higher.
2. Do not use a plastic pipette to transfer the formic acid. The concentrated acid will leach plasticizers out of the plastic and cause a large late eluting peak.
3. Never wash mobile phase bottles with soap or detergent. This causes high background and can never be rinsed out completely. If a bottle is accidentally washed with soap or detergent, discard it or use it for non-LC/MS assays.

4. The concentration of the lowest calibrator is assigned as the lower limit of quantitation (LLOQ), and the highest calibrator concentration is the upper limit of quantitation (ULOQ). The analytical range for buprenorphine and norbuprenorphine using this method is 2–200 ng/mL.
5. Batch acceptance criteria: The back-calculated concentrations of each calibrator and QC must be within ±20% (for the LLOQ, ±25%) and ±25% of target, respectively. The analyte retention times of QCs must match within ±0.05 min of the mean retention time in calibrators. The analyte ion ratio in QCs must match within ±20% of the mean ion ratio in calibrators. The analyte response in blank urine may not exceed 30% of the response at the LLOQ.
6. Analyte identification criteria: The retention time for transitions 1 and 2 must be within ±0.05 min of the mean retention time in the calibrators and within ±0.01 min of each other. The ion ratio must be within ±20% of the mean ion ratio in calibrators.

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Chapter 6

Quantitation of Tapentadol by Liquid Chromatography: Tandem Mass Spectrometry

Graham R. Jones and Russell P. Handy

Abstract

Tapentadol is an orally active analgesic with a similar structure to tramadol. Its primary mechanism of action is agonist action on the mu-opioid receptor. The method described here quantitates tapentadol in the whole blood using a matching deuterated internal standard, extraction via a protein "crash" with acetonitrile, followed by analysis using liquid chromatography-tandem mass spectrometry.

Key words Tapentadol, Deuterated, Mu-opioid, LC-MS/MS

1 Introduction

Tapentadol is a synthetic mu-opioid agonist with a similar structure to tramadol and is used to treat moderate to severe acute pain in patients 18 years of age or older. It is available in the USA as 50–100 mg normal-release tablets and 50–250 mg extended-release tablets. Recommended doses for adults are 50–100 mg every 4–6 h or 50–250 mg twice daily for the extended release form, up to a daily maximum recommended dose of 600 mg. Tapentadol has an estimated half-life of 3–7 h, a volume of distribution of 6–9 L/kg, and relatively low protein binding (Fb 0.20) [1, 2].

Tapentadol undergoes extensive first-pass metabolism by phase 2 glucuronidation to form the O-glucuronide. After oral administration 70% of the dose is excreted in the urine (55% O-glucuronide and 15% sulfate). Only about 3% of tapentadol is excreted unchanged in the urine. Phase 1 oxidative metabolism via CYP2C9 and CYP2C19 is a minor metabolic route, forming primarily N-desmethyltapentadol. None of the metabolites of tapentadol appear to have analgesic activity [2]. Therefore this analytical procedure only measures the active parent drug.

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2 Materials

2.1 Solvents (See Note 1)

1. 50:50 methanol/water (v/v). Add 100 mL methanol to a graduated cylinder. Fill to 200 mL with deionized water and mix. Store at room temperature.
2. Mobile phase A: 0.1% v/v formic acid in deionized water. Add 1 mL of formic acid to a 1 L graduated cylinder. Fill to volume with deionized water and mix.
3. Mobile phase B: acetonitrile.
4. Working standard A: 25 μg/mL tapentadol. Add 0.125 mL of 1 mg/mL tapentadol methanolic stock to a 5 mL volumetric flask. Fill to volume with 50:50 methanol:water. Mix well and store at 4 °C.
5. Working standard B: 2.5 μg/mL tapentadol. Add 0.5 mL of working standard A to a 5 mL volumetric flask. Fill to volume with 50:50 methanol:water. Mix well and store at 4 °C.
6. Working internal standard: 2.5 μg/mL tapentadol-D3. Add 0.125 mL of 0.1 mg/mL tapentadol-D3 methanolic stock to a 5 mL volumetric flask. Fill to volume with 50:50 methanol: water. Mix well and store at 4 °C.
7. Quality control: 0.5 μg/mL tapentadol. Add 12.5 μL of 1 mg/mL tapentadol methanolic stock to a 25 mL volumetric flask. Fill to volume with drug-free whole blood and mix well. Aliquot into 1 mL vials and freeze at ≤ -20 °C (*see* **Note 2**).

2.2 Supplies and Equipment

1. 10 mL glass tubes
2. 1 mL autosampler vials
3. Vortexer
4. Centrifuge with adaptor for 10 mL tubes
5. Triple quadrupole mass spectrometer using electrospray ionization source, e.g., Agilent model 6410 with 12,000 series LC system, or similar
6. Analytical column: Agilent Poroshell SB-C18, 2.1 × 100 mm, 2.7 μm

3 Methods

3.1 Preparation of Working Standards and Unknown Samples

1. Appropriately label a set of 10 mL glass tubes and a set of 1 mL autosampler vials. Label for one tube for each standard, blank and control, and two tubes for each specimen (*see* **Note 3**).
2. Add 0.5 mL drug-free whole blood to each 10 mL tube for the blank and standards. Add 0.5 mL of the control to the

Table 1
Spiking scheme for calibration standards

Final conc. tapentadol (mg/L)	0.025	0.05	0.1	0.25	0.5	1	2.5	5
Working standard A (μL)	–	–	–	–	10	20	50	100
Working standard B (μL)	5	10	20	50	–	–	–	–

appropriate tube. Add 0.5 mL of specimen, in duplicate, to the appropriate specimen tubes.

3. To the standard tubes, add the working standard according to Table 1.
4. Add 0.050 mL of working internal standard to each tube. Vortex and allow to stand 10 min.
5. Add 0.5 mL of 50:50 methanol/water to each tube. Vortex to mix.
6. Add 2 mL acetonitrile to each tube while vortexing, and continue to vortex for 10–15 s. Allow to sit for 10 min and then re-vortex briefly. Centrifuge at 2000×*g* for 5 min.
7. Transfer an aliquot of supernatant to a 1.5 mL autosampler vial. Cap tightly.
8. Inject 0.5 μL on the LC-MS/MS.

3.2 Analysis

1. Place the extracts on the autosampler in the following order: blank, calibrators in order of lowest to highest concentrations, blank (can reinject from same vial), QC sample, unknown samples, and 0.5 mg/L calibrator (reinject from same vial) (*see* **Note 4**).
2. Autosampler parameters cool to constant 4 °C.
3. Injection volume 0.5 μL.
4. Column temperature 45 °C.
5. Flow rate 0.5 mL/min.
6. Mobile phase program (*see* **Note 5**):
   (a) 0.0 min: mobile B ratio 10%
   (b) 4.0 min: mobile B ratio 50%
   (c) 6.0 min: mobile B ratio 50%
   (d) 6.01 min: mobile B ratio 95%
   (e) 8.5 min: mobile B ratio 95%
   (f) 8.51 min: mobile B ratio 10%
   (g) 14.0 min: mobile B ratio 10%
7. Ion source parameters: gas temp = 350 °C, gas flow 11 L/min; nebulizer = 35 psi, capillary voltage 4000 V.

Table 2
Analytical and detection conditions for tapentadol and the deuterated internal standard

Compound	Ret. time (min)	Precursor ion (*m/z*)	Quantifier product ion (*m/z*)	Quantifier collision energy (V)	Qualifier #1 product ion (*m/z*)	Qualifier #1 collision energy (V)	Qualifier #2 product ion (*m/z*)	Qualifier #2 collision energy (V)
Tapentadol	3.6	222.2	107	24	121	18	77	57
Tapentadol-D3	3.6	225.2	107	24	121	18	77	57

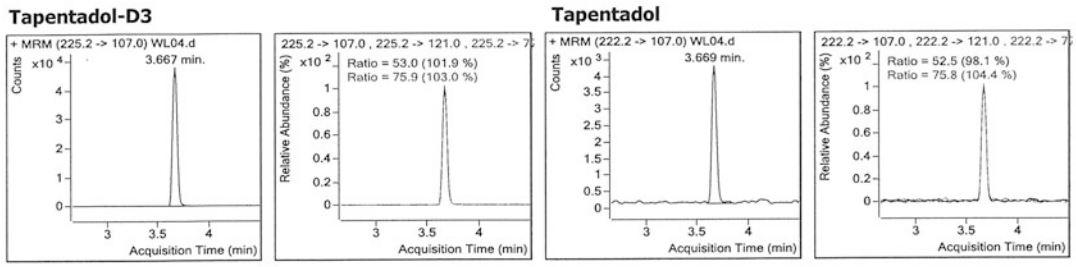

Fig. 1 MRM chromatograms of tapentadol and tapentadol-D3, showing the quantifying ion transitions 222.2 to 107 and 225.2 to 107, respectively, and the corresponding qualifying ions of 121 and 77

8. Mass spectrometer parameters: Compound-dependent parameters are listed in Table 2 (*see* **Note 6**).
9. Figure 1 shows the chromatography of a 0.025 mg/L calibrator containing tapentadol and the internal standard. One quantitation transition and two qualifying transitions are shown for each compound (*see* **Note 7**).

4 Notes

1. Unless otherwise stated, all reagents are of HPLC or analytical grade. Formic acid is 98–100%.
2. Use a different source of tapentadol solution than that used for the calibrators.
3. Case blood samples are run in duplicate as a repeatability check for difficult postmortem blood samples.
4. Sample order is at the discretion of the user. An unextracted standard may be injected prior to the main run in order to verify condition, including the retention time window. An extracted blank sample is injected after the highest calibrator to verify lack of carryover. If applicable, for long runs, a

calibrator is reinjected after every ten samples as a calibration stability check.

5. The original procedure was developed to quantify tramadol and methadone as well as tapentadol, which is why the LC gradient is more complex than might be required to quantify tapentadol alone.
6. Quantifier and qualifier multiple reaction monitoring (MRM) transitions are based on a single precursor ion for each compound. Mass spectrometry parameters will vary between instrument and manufacturers and must be optimized for the specific LC-MS/MS instrument used.
7. Percent accuracy was established in whole blood calibrations ($n = 3$) from 0.025 mg/L (112.9%, 1.7% CV) and 5.0 mg/L (99.8%, 0.3% CV) and independently prepared whole blood controls ($n = 15$) 0.5 mg/L (98.8%, 3.4% CV). Recovery spikes into postmortem case blood ($n = 10$) averaged 96.2% (1.9% CV) at 0.075 mg/L and 100.1% (1.65% CV) at 4.0 mg/L. Ionization efficiency of tapentadol extracted from the whole blood ($n = 5$) was 101.4% (1.8% CV) compared with extraction from DIW ($n = 5$). QC samples run over a 12-month time period from a single frozen batch showed excellent stability (mean 0.48 mg/L, 5.8% CV; $n = 14$).

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Chapter 7

Therapeutic Drug Monitoring of Lacosamide by LC-MS/MS

He Sarina Yang and Leslie Edinboro

Abstract

High-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) has become a primary analytical methodology in therapeutic drug monitoring of antiepileptic drugs (AEDs). To demonstrate the utility of LC-MS/MS in measuring drug concentrations in serum or plasma, analysis of lacosamide (Vimpat™) is discussed in this chapter. Lacosamide is an example of the newer-generation AEDs. The drug is extracted by protein precipitation and dilution of the serum specimen. A small volume of the extracted specimen is injected into a reversed-phase chromatography column, and lacosamide is identified by positive electrospray ionization (ESI) mass spectrometry in the multiple reaction monitoring (MRM) mode, which provides selectivity for quantitative analysis. A deuterated internal standard is used to correct for any loss of analyte during the process of extraction and analysis. A seven-point calibration curve and two levels of quality controls are included in each batch.

Key words Antiepileptic drugs, Lacosamide, LC-MS/MS, Therapeutic drug monitoring

1 Introduction

In the last 20 years, 14 so-called new generation of AEDs have entered the market, including eslicarbazepine, felbamate, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, rufinamide, stiripentol, tiagabine, topiramate, vigabatrin, and zonisamide [1]. Compared to the first-generation AEDs, the newer agents generally have wider therapeutic ranges and fewer serious adverse effects. However, the pharmacokinetics of the new AEDs show significant interindividual variability due to sex, age, race, hepatic metabolism, renal function, and concomitant medications [1]. Therapeutic drug monitoring (TDM) is of particular importance in the clinical management of AED therapy, as therapeutic and toxic effects of AEDs have been found to be related to serum concentration [2]. TDM of new antiepileptic drugs can be of great benefit in the treatment of seizure disorders and in prevention of adverse drug effects. TDM can also aid in individualizing therapy, adjusting for variable or nonlinear pharmacokinetics, and managing

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special populations such as pregnant women. In addition, clinicians rely upon TDM to assess patient compliance and to prevent drug misuse, particularly in patients with psychiatric disorders [3].

In this chapter, lacosamide high-performance liquid chromatography-mass spectrometry (LC-MS/MS) analysis is used as an example of a procedure for quantifying new-generation AEDs in serum or plasma; analysis of most of the other new-generation AEDs was presented in the first edition of this book. Lacosamide is a novel functionalized amino acid that is used as an adjunct therapy for the treatment of partial-onset seizures and focal epilepsies [4]. It is also being investigated as a treatment for diabetic neuropathic pain [5]. Lacosamide is thought to have dual mechanisms of action [6]. It selectively enhances slow inactivation of voltage-gated sodium channels, which stabilizes hyper-excitable neuronal membrane and inhibits neuronal firing. In addition, it modulates the collapsing response mediator protein-2 (CRMP-2). The role of CRMP-2 in seizures has not been fully elucidated; however, its expression is altered in epilepsy and other neurodegenerative disorders. Lacosamide has low plasma protein binding (<15%) and minimal clinically significant drug-drug interactions. Overall, lacosamide exhibits predictable pharmacokinetics with no clinically significant differences in pharmacokinetics between children, young adults, and elderly patients [1].

Automated immunoassay methods have been widely used for TDM of anticonvulsants since the 1980s. However, antibodies used in these methods may cross-react with metabolites of the drug in question [7]. Moreover, immunoassays are not available for many newer-generation AEDs [8]. In recent years, LC-MS/MS has become the primary analytical methodology in therapeutic monitoring of newer AEDs. Compared to immunoassays, LC-MS/MS offers superior sensitivity, specificity, accuracy, and throughput in measuring the concentration of drugs in biological samples. Only a small amount of specimen (100–300 μL) is needed for analysis. Here, we describe a detailed lacosamide LC-MS/MS method used in a large clinical laboratory.

2 Materials

2.1 Internal Standard, Calibrators, Controls, and Buffers

1. Internal standard working solution: 5 μg/mL lacosamide-^{13}C, D_3. Add 100 μL of 1 mg/mL lacosamide-^{13}C, D_3 stock solution to a 20 mL volumetric flask. Fill to volume with methanol and mix well. Store at 2–8 °C for 1 month or − 10 to −30 °C for 6 months.
2. 50% methanol. Add 5 mL of methanol to a 10 mL volumetric flask. Fill to volume with clinical laboratory reagent water

(CLRW) and mix. Prepare fresh prior to preparation of standards.

3. 200 µg/mL lacosamide standard working solution: Add 1 mL of 1 mg/mL lacosamide stock solution to a 5 mL volumetric flask. Fill to volume with 50% methanol and mix well. Prepare fresh prior to preparation of standards.
4. Calibrator 1: 0.5 µg/mL lacosamide. Add 25 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months (*see* **Note 1**).
5. Calibrator 2: 1 µg/mL lacosamide. Add 50 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months.
6. Calibrator 3: 2 µg/mL lacosamide. Add 100 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months.
7. Calibrator 4: 4 µg/mL lacosamide. Add 200 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months.
8. Calibrator 5: 8 µg/mL lacosamide. Add 400 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months.
9. Calibrator 6: 10 µg/mL lacosamide. Add 500 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months.
10. Calibrator 7: 20 µg/mL lacosamide. Add 1000 µL of lacosamide standard working solution to a 10 mL glass volumetric flask containing approximately 5 mL of drug-free serum. Fill to volume with drug-free serum. Aliquot 1 mL into 1.5 mL vials and store frozen. Stable for 6 months.
11. Quality control 1: 5 µg/mL lacosamide. Reconstitute lyophilized serum quality controls (UTAK Laboratories Bi-Level AED II serum toxicology control or similar), by adding exactly 5 mL of CLRW using a volumetric pipette, and mix well on a

shaker. Reconstituted control material is stored at 2–8 °C and is stable for 25 days after reconstitution (*see* **Note 2**).

12. Quality control 2: 20 μg/mL lacosamide. Reconstitute lyophilized serum quality controls (UTAK Laboratories Bi-Level AED II serum toxicology control or similar), by adding exactly 5 mL of CLRW using a volumetric pipette, and mix well on a shaker. Reconstituted control material is stored at 2–8 °C and is stable for 25 days after reconstitution.
13. Mobile phase A: 0.1% formic acid in water. Add 1.0 mL of formic acid to 999 mL of CLRW. Stable at room temperature for 1 month.
14. Mobile phase B: 0.1% formic acid in acetonitrile. Add 1.0 mL of formic acid to 999 mL of acetonitrile. Stable at room temperature for 1 month.
15. Autosampler wash solution: 50% methanol in water. Add 500 mL of methanol to 500 mL of water. Stable in room temperature for a month.

2.2 Supplies and Analytic Equipment

1. Agilent 1200 series pump system operating in laminar flow mode (or equivalent).
2. Applied Biosystems API 3200 mass spectrometer (or equivalent).
3. Sciex Analyst® software.
4. Restek guard column.
5. Restek Pinnacle DB Biphenyl 5 μm 50 x 2.1 mm column.
6. Allegra X-15R benchtop centrifuge, Beckman Coulter.
7. Autosampler Vials-Robo Type 1, Class A (or equivalent).
8. Flat-bottom glass inserts (250 μL).
9. Vials with caps and septa.
10. Vortex mixer.
11. Eppendorf microcentrifuge or equivalent.
12. Tubes, Eppendorf 1.5 mL.
13. 2 mL and 1 mL 96-well collection plates.

3 Methods

3.1 Extraction of Lacosamide from Plasma and Serum

1. Pipette 100 μL of each plasma or serum specimen, calibrator, and control to its own 1.5 mL Eppendorf tube or individual well of a 2 mL 96-well plate (*see* **Note 3**).
2. Add 20 μL of internal standard to each tube or well.
3. Add 200 μL of methanol to each vial or well to precipitate proteins. Vortex to mix then centrifuge for 15 min at 4 °C.

Tubes are centrifuged at 18,538 × *g*; 96-well plates are centrifuged at 4000 × *g*.

4. Transfer 150 μL of supernatant off the protein pellet into individual autosampler vials with flat-bottom glass inserts or a new 1 mL 96-well collection plate.
5. Place samples into the autosampler rack (*see* **Note 4**).

3.2 LC-MS/MS Analysis

1. Inject samples in the following order: calibration standards (S1–S7), blank, quality control level 1, quality control level 2, unknown samples, additional quality controls.
2. Injection volume: 10 μL.
3. Column temperature: room temperature (*see* **Note 5**).
4. Gradient times, flow rates, and solution percentages are listed in Table 1.
5. Mass spectrometry source: electrospray ionization positive mode.
6. Source parameters: curtain gas = 20 psi, ion spray voltage = 5500 V, ion source temperature = 550 °C.
7. Two characteristic fragment ions (or transitions) for lacosamide and one transition for the internal standard are shown in Table 2 (*see* **Note 6**).
8. Chromatography of lacosamide and the internal standard are shown in Fig. 1 (*see* **Note 7**).

Table 1
Gradient program

Step	Total time (min)	Flow rate (mL/min)	A (%) [aqueous]	B (%) [organic]
0	0.10	0.75	90	10
1	0.20	0.75	30	70
2	1.90	0.75	10	90
3	2.00	0.75	90	10

Table 2
Mass transitions for lacosamide and internal standard

Substance	Q1 (*m/z*)	Q3 (*m/z*)	Q3 qualifier (*m/z*)
Lacosamide	251.4	91.0	108.0
Lacosamide-^{13}C, D_3	255.4	91.0	--------

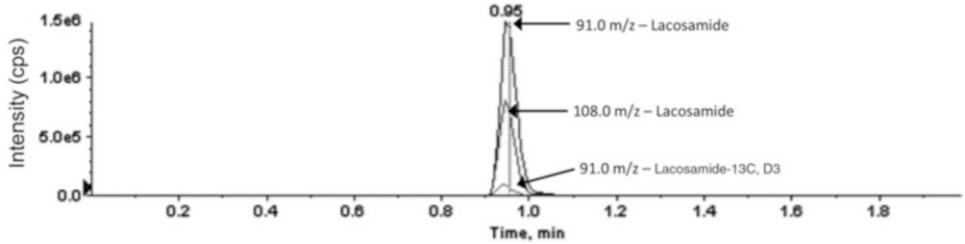

Fig. 1 Example of chromatography for lacosamide (254.1/91.0 and 254.1/108.0) and internal standard lacosamide-^{13}C, D_3 (255.4/91.0)

3.3 Data Analysis

1. Generate calibration curves using a weighted ($1/x$) linear regression curve.
2. Quantitate analyte peaks by normalizing the peak area of the quantifier ion of each compound to the internal standard.
3. Calculate lacosamide concentration from the calibration curve (*see* **Note 8**).
4. Analytical measure range (AMR) of this assay is 0.5 to 20 μg/mL.

4 Notes

1. Calibrators are stable for 6 months if aliquoted in 1 mL vials and kept frozen at − 20 °C.
2. Store reconstituted UTAK controls at 2–8 °C. Once reconstituted they are stable for 25 days.
3. Spiking studies demonstrate that serum, EDTA plasma, and heparin plasma are all acceptable for analysis. Serum stability is at least 5 days at ambient, 14 days refrigerated, and 30 days frozen storage temperature. Hemoglobin, triglycerides, and bilirubin do not significantly interfere with measurement of lacosamide by this method.
4. Post-extraction stability studies indicate that extracted samples are stable for 24 h.
5. Back pressure of column should be monitored. Sudden increase of back pressure may indicate clogging of column, and sudden decrease of pressure may be due to leaking in the HPLC system.
6. To ensure the correct identification of lacosamide, in each batch, the retention time of lacosamide peak should be within ±0.2 s of the established time. The peak area of internal standard should be within 30% of the established value and remain stable within the run. The chromatogram should be symmetrical, well resolved, and Gaussian distributed regarding the morphology, free of tailing or fronting peak, or any interference peak.

7. If a peak shows a sign of having column overload (peak fronting), sample should be repeated at twofold or fourfold dilution to ensure correct quantitation.
8. Using a calibration curve, the relative abundance of a given ion is converted to an absolute amount of the original molecule. Regression coefficient of the calibration curve should be above 0.98. Only one calibrator can be excluded from the curve. If the lowest calibrator is removed, patient samples having results lower than the second lowest calibrator need to repeated; if the highest calibrator is removed, patient samples having results higher than the second highest calibrator need to be repeated.

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Chapter 8

LC-MS/MS Method for the Quantification of the Leflunomide Metabolite, Teriflunomide, in Human Serum/Plasma

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Abstract

Leflunomide is a prodrug that is metabolized to the active metabolite, teriflunomide (A77 1726), to inhibit the enzyme dihydroorotate dehydrogenase and decrease the synthesis of pyrimidine nucleotides for DNA and RNA synthesis. Teriflunomide is primarily used for the treatment of rheumatoid arthritis and multiple sclerosis.

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated to quantify the drug teriflunomide over a concentration range of 5 ng/mL–200 μg/mL in serum or plasma. The calibration curve was divided into two separate overlapping regions of the analytical measurement range, with a high curve and a low curve range. Samples are first analyzed using the high-range calibration curve after a 100-fold dilution of the sample extract. Samples falling below the upper curve region are evaluated again without dilution and quantified, if possible, against the low curve calibration standards. This method can be used to support therapeutic drug monitoring of patients that are administered with leflunomide therapy.

Key words Leflunomide, Teriflunomide, Immunosuppressant, LC/MS/MS

1 Introduction

Leflunomide is a prodrug approved by the FDA in 1998 and brought to market by Sanofi-Aventis under the name Arava®. It is administered largely for the treatment of rheumatoid arthritis and is classified as a disease-modifying antirheumatic drug (DMARD) and functions to slow disease progression [1]. Leflunomide contains an isoxazole ring which is opened nonenzymatically to the active metabolite, teriflunomide. Another drug formulation, marketed as Aubagio®, consists of the ring-opened form and is used for the treatment of active relapsing-remitting multiple sclerosis [2]. Leflunomide has also been prescribed, off-label, for other uses in renal

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transplant patients, treating cytomegalovirus viremia [3], BK virus-associated nephropathy [4], for treatment in psoriatic arthritis [5] and Wegener granulomatosis, systemic lupus erythematosus, sarcoidosis, and more [6].

Teriflunomide inhibits cell proliferation of activated lymphocytes by inhibition of the enzyme, dihydroorotate dehydrogenase, to decrease the synthesis of pyrimidine nucleotides for DNA and RNA synthesis [7, 8]. Once the drug is metabolized, circulating concentrations of leflunomide after oral administration are generally very low in comparison with levels of teriflunomide [9]. In vivo, leflunomide is rapidly and, essentially, completely converted to teriflunomide that has both immunosuppressive and antiviral effects. Studies have shown a correlation between teriflunomide blood concentrations of 40 μg/mL and progressive clearance of BK polyomavirus (BKV) [10, 11]. BKV is a common infection in immunosuppressed renal transplant patients that can lead to failure and loss of grafted kidneys. Teriflunomide reaches a maximum plasma concentration approximately 6–12 h following oral administration [7]. The drug is cleared both in the urine and in the feces (approximately 43% and 48%, respectively) [7]. It has a steady-state volume of distribution (Vd) of just 0.13 L/kg and clearance of approximately 31 mL/h [7].

Both leflunomide and teriflunomide have the same molecular weight, and after collision-induced dissociation of the protonated parent by tandem mass spectrometry, both compounds have the same two most abundant product ions. Consequently, chromatographic separation of leflunomide from teriflunomide is necessary to eliminate the possibility of interference in the quantification of the parent drug and metabolite.

The therapeutic range for teriflunomide is not well established; however serum/plasma concentrations >40 μg/mL tend to correlate with improved patient outcome. The drug is teratogenic; thus, clearance (defined as concentrations <20 ng/mL) is monitored as well. Patient drug concentrations in serum or plasma could range from several hundred μg/mL down to the medical decision point of 20 ng/mL [12]. Here we describe a method that covers a 40,000-fold range, from 5 ng/mL to 200 μg/mL, by the implementation of two separate but overlapping calibration curves. A single extraction procedure is utilized for both the high and low curve ranges, with the exception that samples analyzed for the higher concentration range are diluted 100-fold prior to analysis. Earlier methods for determination of teriflunomide or leflunomide have been described in the literature as using HPLC/UV [9, 13, 14] or LC/MS/MS [15].

2 Materials

2.1 Supplies and Equipment

1. Volumetric glassware and pipettes.
2. Pipette tips.
3. Agilent 1200 series HPLC pump.
4. CTC autosampler.
5. AB Sciex API4000 triple quadrupole mass spectrometer with Analyst quantitation software (ver. 1.5.1). The instrument employs an ESI interface, multiple reaction monitoring (MRM), unit resolution performed in negative ionization mode.
6. Autosampler vials.
7. Analytical column; 2 mm × 10 cm, Luna PFP [2] (pentafluorophenyl) phase on 3 μm particles.
8. Microcentrifuge tubes (1.7 mL).
9. Vortex mixer.
10. Centrifuge (to accommodate microcentrifuge tubes and capable of achieving 207083 rcf).

2.2 Standards and Controls

The high calibration curve consists of standards 6–10; the low calibration curve consists of standards 1–5. Controls for the high curve are D, E, and F; controls for the low curve are A, B, and C.

1. Stock solution: 10 mg/mL teriflunomide in DMSO. Add 10 mg of teriflunomide powder to 1 mL of dimethyl sulfoxide (DMSO). Mix until completely dissolved.
2. Standard 10: 200 μg/mL teriflunomide. Add 5 μL of stock solution to 245 μL of drug-free serum. Vortex to mix. Make fresh daily.
3. Standard 9: 50 μg/mL teriflunomide. Add 40 μL of standard 10–120 μL of drug-free serum. Vortex to mix. Make fresh daily.
4. Standard 8: 10 μg/mL teriflunomide. Add 10 μL of standard 10–190 μL of drug-free serum. Vortex to mix. Make fresh daily.
5. Standard 7: 2 μg/mL teriflunomide. Add 10 μL of standard 10–990 μL of drug-free serum. Vortex to mix. Make fresh daily.
6. Standard 6: 0.8 μg/mL teriflunomide. Add 100 μL of standard 7–150 μL of drug-free serum. Vortex to mix. Make fresh daily.
7. Standard 5: 1 μg/mL teriflunomide. Add 200 μL of standard 7–200 μL of drug-free serum. Vortex to mix. Make fresh daily.
8. Standard 4: 0.5 μg/mL teriflunomide. Add 100 μL of standard 5–100 μL of drug-free serum. Vortex to mix. Make fresh daily.
9. Standard 3: 0.1 μg/mL teriflunomide. Add 25 μL of standard 4–100 μL of drug-free serum. Vortex to mix. Make fresh daily.

10. Standard 2: 0.02 μg/mL teriflunomide. Add 10 μL of standard 4–240 μL of drug-free serum. Vortex to mix. Make fresh daily.
11. Standard 1: 0.005 μg/mL teriflunomide. Add 5 μL of standard 4–495 μL of drug-free serum. Vortex to mix. Make fresh daily.
12. Control F: 170 μg/mL teriflunomide. Use a separate preparation of stock solution than for calibrators. Add 170 μL of stock solution to a 10 mL volumetric flask. Fill to volume with drug-free serum. Vortex mix and aliquot into microcentrifuge tubes. Store at −20 °C or lower until use.
13. Control E: 80 μg/mL teriflunomide. Use a separate preparation of stock solution than for calibrators. Add 80 μL of stock solution to a 10 mL volumetric flask. Fill to volume with drug-free serum. Vortex mix and aliquot into microcentrifuge tubes. Store at −20 °C or lower until use.
14. Control D: 1 μg/mL teriflunomide. Add 125 μL of control E to a 10 mL volumetric flask. Fill to volume with drug-free serum. Vortex mix and aliquot into microcentrifuge tubes. Store at −20 °C or lower until use.
15. Control C: 0.8 μg/mL teriflunomide. Add 100 μL of control E to a 10 mL volumetric flask. Fill to volume with drug-free serum. Vortex mix and aliquot into microcentrifuge tubes. Store at −20 °C or lower until use.
16. Control B: 0.1 μg/mL teriflunomide. Add 12.5 μL of control E to a 10 mL volumetric flask. Fill to volume with drug-free serum. Vortex mix and aliquot into microcentrifuge tubes. Store at −20 °C or lower until use.
17. Control A: 0.02 μg/mL teriflunomide. Add 200 μL of control D to a 10 mL volumetric flask. Fill to volume with drug-free serum. Vortex mix and aliquot into microcentrifuge tubes. Store at −20 °C or lower until use.
18. Internal standard/protein precipitating solution: 333 ng/mL d_4-teriflunomide. Add 30 μL of a 1 mg/mL stock solution of the deuterated internal standard to 30 mL of 1:1, methanol/acetonitrile containing 0.1% formic acid. Allow the solution to mix for 30 min. at room temperature (20–25 °C) prior to storage.

2.3 Solutions

1. Mobile phase a: 0.1% formic acid in deionized water. Add 1 mL of formic acid to 999 mL of deionized water in a graduated cylinder. Mix.
2. Mobile phase B: 0.1% formic acid in 1:1:18 water/methanol/acetonitrile. Add 1 mL of formic acid, 50 mL of deionized water, and 50 mL of methanol to a 1 L graduated cylinder. Fill to volume with acetonitrile and mix.

3. Double blank solution: 0.1% formic acid in 1:1 methanol/acetonitrile. Add 1 mL formic acid to 500 mL of methanol and 500 mL of acetonitrile to a 1 L graduated cylinder, and mix.
4. Dilution solution: 0.1% formic acid in 25% water, 75% 1:1 methanol/acetonitrile. Add 1 mL of formic acid, 250 mL of deionized water, 375 mL of methanol, and 375 mL of acetonitrile to a 1 L graduated cylinder, and mix.
5. Autosampler needle wash 1; 4:1 methanol/water containing 0.1% trifluoroacetic acid. Add 1 mL of trifluoroacetic acid and 200 mL of deionized water to a 1 L graduated cylinder. Fill to volume with methanol and mix.
6. Autosampler needle wash 2; 2:3 methanol/water. Add 400 mL of methanol to 600 mL of deionized water in a graduated cylinder and mix.
7. Test injection solution: 100 pg/μL teriflunomide and leflunomide in 1:3 water/acetonitrile. Combine 100 μL each of 10 μg/mL solutions of teriflunomide and leflunomide with 2.45 mL of water in a 10 mL volumetric flask. Fill to volume with acetonitrile and mix.
8. Heparinized or EDTA plasma, plain (red top) serum: Collect and process specimens according to standard phlebotomy procedures. Freeze specimens at −20 ° C or colder and transport.

3 Methods

3.1 Sample Preparation

1. Label a microcentrifuge tube for each standard [1–10], control (A–F), patient sample, blank, and double blank (*see* **Note 1**).
2. Add 100 μL of each blank serum, standard, control, and patient serum/plasma to the appropriate microcentrifuge tubes.
3. Add 300 μL of "double blank solution" to the tubes designated double blanks.
4. Add 300 μL of "working internal standard" to all other blanks, calibrators, controls, and patient samples (*see* **Note 2**).
5. Cap tubes and vortex mix for 2 min.
6. Centrifuge for 10 min at 20783 rcf.
7. Prepare samples for injection:
   (a) *High curve*: Transfer 2 μL of each patient, calibrator 6–10, control D-F, and blank supernatant into autosampler vials containing 200 μL of dilution solution. Cap and mix.
   (b) *Low curve*: Transfer 100 μL of each calibrator 1–5, control A–C, blank, and low patient (*see* **Note 3**) supernatant into autosampler vials and cap.

Table 1
Chromatography conditions

Step	Total time (min)	Flow rate (μL/min)	A (%)a	B (%)
0	0.0	300	40	60
1	0.2	300	40	60
2	2.0	300	0	100
3	4.0	300	0	100
4	4.1	300	40	60
5	4.9	300	40	60

[a]A(%) and B(%) refer to the percentages of mobile phases A and B, respectively

Table 2
MS/MS operating parameters

MS/MS parameter table
Curtain gas (CUR): 25 psi
Temperature (TEM):500 °C
Ion source gas 1 (GS1): 35 psi
Ion source gas 2 (GS2): 30 psi
Nitrogen gas is used for both GS1 and GS2
Ihe: ON
IonSpray voltage (IS): −4300 V
Collision gas (CAD): Medium
Declustering potential (DP): −55 V
Entrance potential (EP): −10 V
Detector parameters (negative)
CEM 2500

3.2 Analysis

1. Inject test solution of leflunomide and teriflunomide (*see* **Note 4**).
2. Inject 3 μL of each sample in sequence.
3. Inject high curve samples first. Determine if any patients need to be run on the low curve, then prepare as described above and inject if required.
4. Analytical column temperature: 24 °C.
5. LC gradient is shown in Table 1.
6. MS parameters are as shown in Tables 2 and 3 (*see* **Note 5**).

Table 3
List of precursor and product ions for teriflunomide and internal standard

	Precursor ion	Product ion	Dwell (msec)	Collision energy (eV)	Collision cell exit potential
Teriflunomide	269.1	82.0	50	17	5
	269.1	160.0	50	26	2
d_4-teriflunomide	273.1	82.0	50	27	5
	273.1	164.0	50	34	2

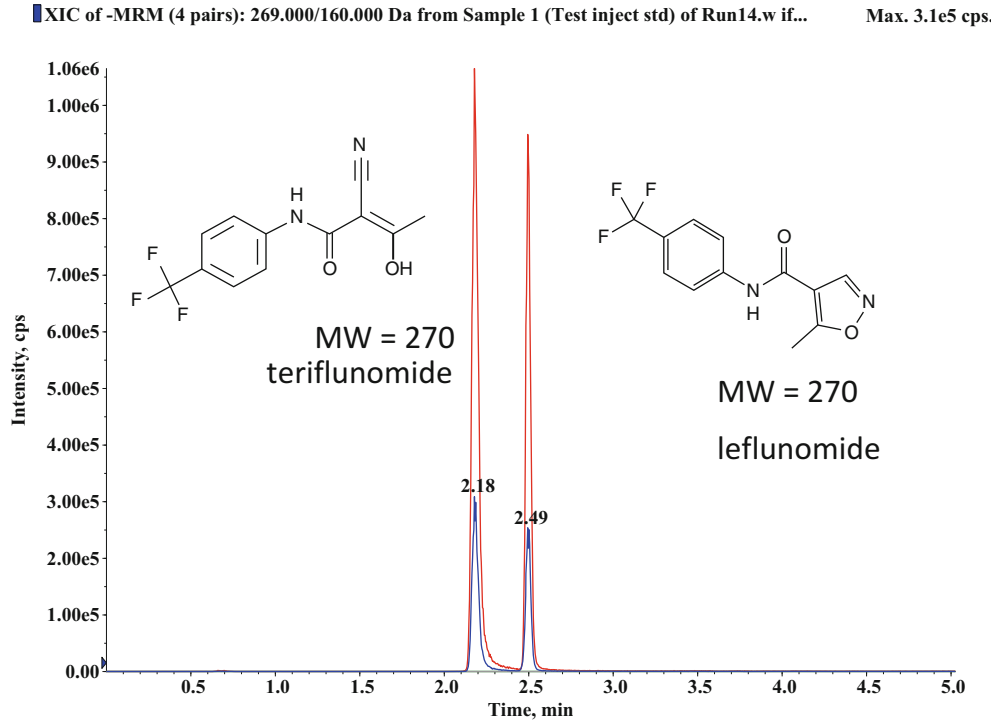

Fig. 1 Extracted ion current chromatograms of teriflunomide in standard 1 (5 ng/mL), and internal standard (d_4 teriflunomide), showing two transitions for each

7. Collect and analyze data using AB Sciex Analyst® software (ver 1.5.2). Example extracted ion current profiles for the low curve standard 1 and internal standard are shown in Fig. 1.
8. Integrate peak height and perform linear regression analysis of the calibration standards with $1/x^2$ weighted regression of peak height ratio vs. analyte concentration. The unknown peak height value divided by the internal standard peak height value gives the ratio.

4 Notes

1. For each curve range, both a *blank* and *double blank* sample are prepared. A *blank* is analyzed as the first and last injection of each sequence and consists of a known negative serum sample. A *double blank* is analyzed after each high standard. A "double blank" sample is one prepared in identical fashion to the ordinary *blank* but in the absence of internal standard. Placing this sample after the high calibration standard allows one to distinguish between autosampler carryover and unlabeled analyte that may be contributed through the internal standard addition as an impurity.
2. Prepare the low and high calibration curve standards, all quality control samples and patient samples in the same batch. Prepare each patient sample with 100× dilution, as described, and evaluate against the high curve calibration standards. If within the high calibration curve region, report the determined value. Samples above 200 μg/mL must be extracted a second time with appropriate matrix dilution. Patient samples below 0.8 μg/mL (the lower limit of quantitation of the high curve) can be prepared according to instructions for the low curve, then analyzed along with the low curve calibrators and quality control samples. Report the resulting value as appropriate.
3. The concentration of internal standard utilized is equivalent to 1 μg/mL in the plasma/serum sample. This concentration is near the low end of the upper curve and at the high end of the lower curve. It is possible to use this same solution (concentration) for both curves since there is a lack of a significant analyte isotope interference with the IS and because only a small amount of unlabeled analyte is present in the IS. We discuss this topic elsewhere in detail [16].
4. A test injection is made with a solution of teriflunomide and leflunomide to verify instrument performance (sensitivity and retention time) and chromatographic separation (resolution) prior to each batch of samples. Results are compared with historical values, and a judgment is made regarding system suitability for patient sample analysis.
5. Although the parent drug, leflunomide, is generally found only at very low concentrations, if at all, it is separated chromatographically from the metabolite, teriflunomide, due to the fact that the two have both the same precursor and product ion masses.

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Chapter 9

Analysis of Tryptic Peptides from Therapeutic Monoclonal Antibodies Using LC-MS/MS

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Abstract

Immunotherapies are a hot topic, with the potential to impact our understanding of the immune system and treat a diverse array of conditions. Therapeutic monoclonal antibodies (mAbs) are part of this revolution, and clinical chemists are aware of the success of the biologic drugs. Antibodies are not just immunoassay reagents anymore but are also present in clinical serum samples from more and more patients each day. The clinical laboratory will have many roles as mAb therapies expand, including the development of new assays to differentiate a mAb from an endogenous, disease-causing clone and monitoring therapeutic drugs for better patient outcomes and assessing for the loss of response to therapy.

Therapeutic mAbs use has expanded significantly in the last 5 years, and depending on their target or their concentration, they may impact routine clinical testing for patients. Optimizing therapy during the induction phase to keep the mAb concentrations above certain thresholds has proven to be associated with improved responses and better outcomes in chronic conditions such as inflammatory bowel disease. This chapter will describe a LC-MS/MS protocol for analysis of tryptic peptides unique to infliximab (clonotypic peptides) for quantitation of the mAb. The protocol can be adapted to other mAbs with similar outcomes and is a useful, relatively simple strategy for measurement of mAbs.

Key words LC-MS/MS, Infliximab, Therapeutic monoclonal antibodies, Trypsin, Tryptic peptides, Method development

1 Introduction

Therapeutic monoclonal antibodies (mAbs) are a relatively novel, growing field in the pharmaceutical industry, with over $100 billion dollars in sales across the world in 2017. Intact therapeutic mAbs are typically modeled after the human IgG class of immunoglobulins (Igs): homodimers consisting of two identical glycosylated heavy chains (50–70 kDa) and two identical light chains (22–24 kDa) linked together by disulfide bonds. The Ig is further segmented at the hinge region by function, with the Fab (fragment antigen binding) representing the upper segment of the protein

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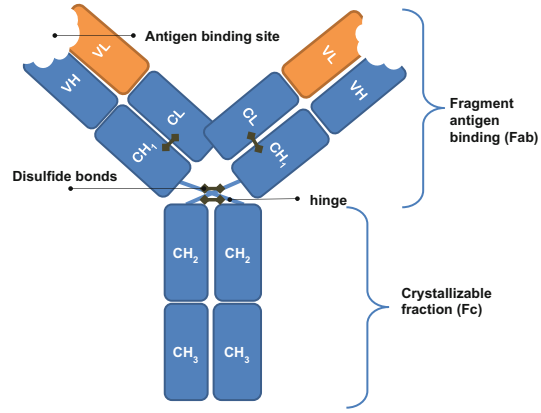

Fig. 1 Immunoglobulin structure. Each immunoglobulin (Ig) consists of two identical glycosylated heavy chains (50–70 kDa) and two identical light chains (22–24 kDa). The light chains are linked to the heavy chains by a single disulfide bond, while the heavy chains are linked together by two or more disulfide bonds depending on the Ig isotype and/or subclass. The Ig is further segmented at the hinge region by function, with the Fab (fragment antigen binding) representing the upper segment of the protein and the Fc (crystallizable fraction) portion representing the lower segment of the protein. VL, variable region of the light chain; VH, variable region of the heavy chain; CL, constant region of the light chain; CH1, CH2, and CH3, constant regions 1, 2, and 3 of the heavy chain

and the Fc (crystallizable fraction) portion representing the lower segment of the protein (Fig. 1).

The two Fab arms contain the N-terminal portion of the heavy chain and are associated with either a kappa or lambda light chain. Together the N-terminal portions of the heavy and light chains form the variable region, which contains the antigen-binding complementary determining region (CDR). The C-terminal portion of each heavy and light chain contains the constant region. Therapeutic mAbs are usually of the IgG kappa isotype, predominantly of the IgG1 subclass, with a few being IgG2, IgG4, or hybrids. When using mass spectrometry to characterize and quantify mAbs, the variable region is the target, and its uniqueness is compared to the endogenous polyclonal Ig repertoire [1].

Therapeutic mAbs have unique characteristics when compared to small molecule drugs. They do not undergo traditional phase I or phase II metabolism or elimination via cellular transporters. Instead, the Fc portion is recognized by receptors on the surface of endothelial cells that internalize the Igs, leading to lysosomal degradation [2]. The pharmaceutical industry has invested in several modifications (Fc engineering) to increase mAb half-lives in circulation in order to increase efficacy. Most mAbs in clinical use have half-lives of a week or longer, resulting in long dosing intervals (2–8 weeks). Intravenous administration allows for application of larger volumes, lower immunogenicity, and higher bioavailability,

whereas more convenient subcutaneous injections will work well for smaller volumes [1].

Most of the clinical indications for monitoring mAbs concentrations are related to loss of response to therapy and poor therapeutic efficacy. Clinical tests for infliximab, adalimumab, certolizumab, vedolizumab, and eculizumab are available commercially in the United States, and there are many more in the pipeline. Infliximab and adalimumab are among the top 5 mAb best sellers, and serum measurement of mAb concentrations at trough is increasingly becoming part of routine patient management. Especially considering the high cost of therapy, which can vary from $15,000 a year for TNF inhibitors to $500,000 a year for complement inhibitors, both the healthcare system and patients can significantly benefit from higher rates of success and fewer treatment failures. Methodologies for measurement of infliximab and adalimumab vary widely and include both mAb quantitation and the measurement of immunogenicity, i.e., the presence of anti-drug antibodies. Immunogenicity is invariably assessed using immunoassays, due to its heterogeneous nature. The mAb quantitation may be performed using immunoassays, cell-based assays [3, 4], liquid chromatography [5], or mass spectrometry [6]. Currently mass spectrometry plays a central role in the routine quality control of therapeutic mAb production [7–9], and new mass spectrometry-based methodologies for monitoring are continuously appearing in the literature [1, 10–13].

1.1 Tryptic Peptide Methods

In order to quantify mAbs using mass spectrometry, the mAb of interest must first be differentiated from the very similar polyclonal background of over 1 g/dL of endogenous human Igs in serum. Historically, proteins have been quantitated by LC-MS/MS using specific tryptic fragments (referred to as "proteotypic" peptides) [14]. Multiple tryptic peptides can be quantitated at the same time, as shown for the quantitation of IgG subclasses in serum by LC-MS/MS [15]. For chimeric mAbs, whose entire variable region is of animal origin, such as infliximab and rituximab, trypsin digestion is possible as the nonhuman variable region is large (>250 amino acids). This increases the likelihood of finding unique signature peptides on the light chain and/or heavy chain which is specific to that mAb and not found in the human polyclonal background.

Not every mAb will be amiable to the digest method especially as newer mAbs are humanized (only small portions of the variable region are of animal source, and they are engrafted onto a human framework, such as eculizumab) or even fully human (genetically engineered antibodies generated by phage display libraries, such as adalimumab). Experiments to find a peptide specific for adalimumab, for instance, failed to detect a tryptic peptide not found in the polyclonal serum background (data not published). For other mAbs, sensitivity of the method will be the limiting factor. For

example, while rituximab is a chimeric IgG1 mAb similar to infliximab, it was found that the tryptic method did not allow for the sensitivity needed for a clinical assay, without pre-analytical immunoenrichment.

One of the challenges with the development of a tryptic method for large proteins such as mAbs is the standardization of the digest. Below we describe a method for LC-MS/MS quantitation of tryptic peptides unique to infliximab, a chimeric (70% human/30% murine) IgG1 kappa monoclonal antibody targeting tumor necrosis factor (TNF) alpha. Briefly, the protocol utilizes a simple and relatively inexpensive purification or Ig-enrichment step using the Ig fraction after saturated ammonium sulfate precipitation. The mixture is then treated with trifluoroethanol (TFE), dithiothreitol (DTT), and iodoacetamide (IAA) to unfold the protein, reduce the disulfide bonds between the heavy and light chains, and prevent their reformation. Trypsin is then added to cut the chains into specific peptides: two of these peptides being the infliximab-unique peptides from the light and heavy chain variable regions of the mAb (Fig. 2). After addition of isotopically labeled

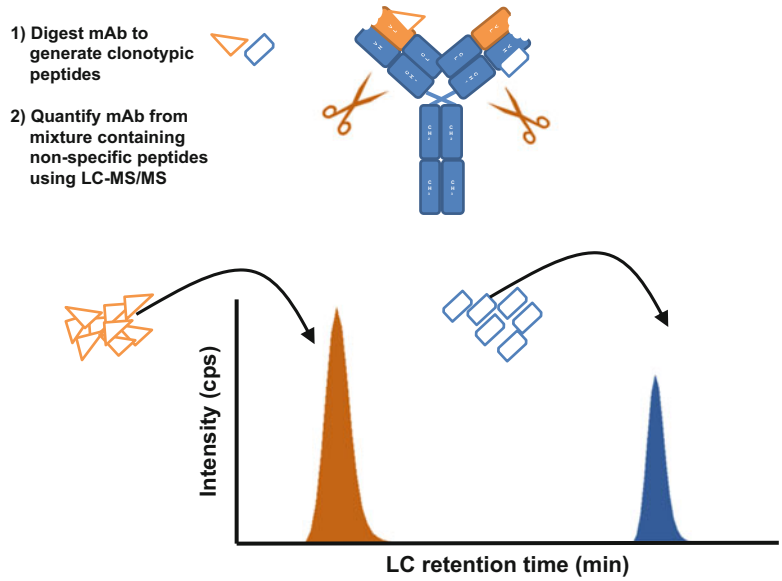

Fig. 2 Trypsin digestion will generate unique peptides for quantitation. Pre-analytical steps will promote denaturation (protein unfolding) and reduction (cysteine reduction breaks disulfide bond connecting mAb light and heavy chains), and alkylation of cysteines prevents disulfide bond from reforming. Subsequently, digestion by trypsin cleaves the intact immunoglobulin into smaller peptides. The peptide mixture is separated by liquid chromatography before analysis by tandem mass spectrometry. Peptides specific to the variable region of the mAb either on the light chain or heavy chain, which do not cross-react with human sequences, are used to quantitate the mAb

peptide retention time standards, the mixture is subjected to reverse-phase C8 liquid chromatography and selective reaction monitoring using a triple quadrupole mass spectrometer. Fragment ions from the infliximab-specific peptides are monitored and compared to a standard curve for quantitation [6]. This method can be adapted and translated to other mAbs with similar outcomes.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Reagents should be prepared at room temperature, unless otherwise noted.

2.1 Reagents and Mobile Phases

1. 2,2,2-Trifluoroethanol (TFE): When handling it, wear gloves and pipette under a hood. Store at room temperature.
2. 50 mM ammonium bicarbonate: Weigh out 791 mg of ammonium bicarbonate and dissolve in 200 mL of water, mixing well at room temperature. Store at room temperature.
3. Saturated ammonium sulfate solution: Weigh out 150 g of ammonium sulfate. Add 200 mL of water. Keep solution at room temperature. Mix periodically over 8 h or more to ensure solution is saturated and crystals are still visible (*see* **Note 1**). Immediately before use, mix solution for 30 min using a stir plate. Store at room temperature.
4. 200 mM dithiothreitol (DTT). Weigh out 30.85 mg of DTT. Add 1 mL of 50 mM ammonium bicarbonate to dissolve. This solution can be scaled up for larger batches; each sample requires 0.01 mL of DTT. This reagent should be prepared fresh daily; do not store (*see* **Note 2**).
5. 200 mM iodoacetamide (IAA). Weigh out 37 mg of IAA. Add 1 mL of 50 mM ammonium bicarbonate to dissolve the powder. This solution can be scaled up for larger batches; each sample requires 0.02 mL of IAA. This reagent should be prepared daily. Once ready, wrap the solution in aluminum foil (*see* **Note 3**) and keep it at room temperature.
6. 1 mg/mL trypsin from bovine pancreas. Weigh out 5 mg of trypsin. Dissolve in 5 mL of 50 mM ammonium bicarbonate. The solution should be prepared fresh daily (*see* **Note 4**).
7. 1% formic acid. To a 25 mL volumetric flask, add 25 μL of formic acid. Bring up to volume with water, mixing well. The solution should be stored at room temperature.
8. Mobile phase A: 0.1% formic acid in water. In a 2 L reagent bottle, add 2 L of water. Then, add 2 mL of formic acid, mixing well. The solution should be stored at room temperature and discarded after 1 week of use.

9. Mobile phase B: 0.1% formic acid in acetonitrile. In a 2 L reagent bottle, add 2 L of acetonitrile. Then, add 2 mL of formic acid, mixing well. Degas the solution. Store at room temperature and discard after 1 week of use.

2.2 Standards and Controls

Calibrators (standards) and controls are prepared by spiking infliximab into normal pooled human serum (*see* **Note 5**). Prepare standards using volumetric flasks and good laboratory pipetting practices.

1. 10 mg/mL Infliximab stock solution: Obtain the pharmaceutical preparation (trade name Remicade, Janssen Biotech) from a pharmacy. Keep refrigerated at 4 °C until the day of reconstitution. The infliximab vial contains 100 mg lyophilized powder of infliximab. Add 10 mL of water using a volumetric pipette to the entire vial contents to obtain a concentration of 10 mg/mL (*see* **Notes 6** and **7**).
2. 100 μg/mL infliximab standard. Add 1 mL of 10 mg/mL infliximab stock solution to a 100 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
3. 50 μg/mL infliximab standard. Add 25 mL of the 100 μg/mL infliximab standard to a 50 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
4. 20 μg/mL infliximab standard. Add 10 mL of the 100 μg/mL infliximab standard to a 50 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
5. 10 μg/mL infliximab standard. Add 5 mL of the 100 μg/mL infliximab standard to a 50 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
6. 5 μg/mL infliximab standard. Add 2.5 mL of the 100 μg/mL infliximab standard to a 50 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
7. 2 μg/mL infliximab standard. Add 1 mL of the 100 μg/mL infliximab standard to a 50 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.

8. 1 μg/mL infliximab standard. Add 1 mL of the 100 μg/mL infliximab standard to a 100 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 350 μL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
9. Low quality control (QC): 3 μg/mL infliximab. Add 30 μL of the 10 mg/mL infliximab stock solution to a 100 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 1 mL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C (*see* **Note 8**).
10. Medium QC: 10 μg/mL infliximab. Add 100 μL of the 10 mg/mL infliximab stock solution to a 100 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 1 mL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
11. Medium-high QC: 25 μg/mL infliximab. Add 250 μL of the 10 mg/mL infliximab stock solution to a 100 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 1 mL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
12. High QC: 80 μg/mL infliximab. Add 800 μL of the 10 mg/mL infliximab stock solution to a 100 mL volumetric flask. Bring to volume with normal (drug-free) human serum. Aliquot 1 mL into microcentrifuge tubes; stable frozen for 2 years at <−60 °C.
13. 0.2 mg/mL horse IgG digestion standard working solution: Pipette 1 mL of 10 mg/mL stock horse IgG solution into a 50 mL volumetric flask and bring to volume with water. Transfer contents to a 50 mL Falcon tube and store frozen at -20 °C (*see* **Note 9**).
14. Isotopically labeled tryptic peptides (^{13}C, ^{15}N stable isotopes): Synthesize stock powder of isotopically labeled peptides for the light chain (YASESMSGIPSR) and heavy chain (GLEWVAEIR) of infliximab. A 0.04 nM scale is recommended, using a combination of ^{13}C and ^{15}N on valine (V), isoleucine (I), and proline (P) amino acids of the peptides (*see* Table 1). Keep stock peptides under vacuum at room temperature inside a desiccator.
15. 1 mg/mL isotopically labeled tryptic peptides stock solutions: Weigh out 10 mg of each labeled peptide separately. Add 10 mg of one of the peptides to a 10 mL class A volumetric flask. Bring to volume with 1% formic acid, mixing well. Repeat the procedure with the second labeled peptide. Store frozen at −80 °C.

Table 1
Tryptic peptides and transitions used to quantify infliximab

Peptides	Transitions	DP (V)	CE (V)	CXP (V)
Infliximab heavy chain peptide (1071.57 Da)				
GLEWVAEIR				
-y4 ion	537.1/488.4	70	23	45
-y5 ion	537.1/587.5	70	25	45
GLEWV(+6)AEI(+7)R				
-y4 ion+7	543.4/495.4	40	23	25
-y5 ion+13	543.4/600.5	40	25	25
Infliximab light chain peptide (1283.58 Da)				
YASESMSGIPSR				
-y6 ion	643.2/616.3	78	37	45
-y10 ion	643.2/1050.5	78	27	45
YASESMSGI(+7)P(+6)SR				
-y6 ion+13	649.5/629.6	160	37	45
-y10 ion+13	649.5/1063.7	160	27	45
Horse IgG light chain peptide (1377.74 Da)				
VNNQALPQPIER				
-y4 ion	689.9/514.3	81.4	35.1	45
-y6 ion	689.9/739.4	81.4	35.1	45

Notes: *DP* declustering potential, *V* volts, *CE* collision energy, *CXP* collision cell exit potential

16. 5 μg/mL isotopically labeled standard working solutions: Add 50 μL of the 1 mg/mL YASESMSGIPSR light chain peptide stock solution to a 10 mL volumetric flask. To the same flask, add 50 μL of the 1 mg/mL GLEWVAEIR heavy chain peptide stock solution. Bring to volume with 1% formic acid, mixing well. Transfer contents to a Falcon tube. The working solution is stable for 1 year after prepared when kept frozen at −20 °C.

2.3 Supplies and Instrumentation

1. Disposable culture tubes, 75 × 12 mm, 4.5 mL polypropylene.
2. Pipette tips, various sizes.
3. HandiStep or other repeat pipetter.
4. Eppendorf barrels, various sizes.
5. 96 deep-well 1 mL round microtiter plate.
6. A triple quadrupole mass spectrometer (API5000, AbSciex) or similar is used to monitor multiple ion pairs.

7. A Cohesive LX4 multiplex system (Shimadzu) or similar chromatography apparatus is used for sample separation and introduction into the mass spectrometer.
8. Waters X Bridge C8 3 × 30 mm; 3.5 μm analytical column. Similar C8 columns can be used.
9. Precolumn filter: C8 4 × 2.0 mm.
10. Analyst software from AB Sciex is used for data processing.
11. Orbital shaker.
12. Misonix Ultrasonic Liquid Processor.
13. Benchtop Shaking/Rotating Incubator Scientific Industries Incubator.
14. Beckman Allegra X-30R Tabletop Plate Centrifuge or equivalent.

3 Methods

Briefly, the method consists of sample preparation for an overnight trypsin digestion followed by liquid chromatography separation of peptides and mass spectrometry detection of analytes and standards.

3.1 Saturated Ammonium Sulfate Extraction

1. Label a 75 × 12 mm polypropylene conical tube for each calibration standard, blank (normal human serum, no infliximab), control material, and patient sample.
2. Transfer 100 μL of calibration working standards, blank, controls, and patient samples to appropriately labeled tubes.
3. Add 50 μL of the 0.2 mg/mL horse IgG working solution to all tubes. Vortex for 30 s (*see* **Note 10**).
4. Add 75 μL of saturated ammonium sulfate to each tube, and vortex for 30 s.
5. Centrifuge all samples at 3000×*g* for 10 min.
6. Pipette and discard the supernatant off of the pellet now formed in the tubes.
7. Reconstitute the pellet in 100 μL of 50 mM ammonium bicarbonate. Alternate vortexing for 30 s and resting for 30 s, for 10 min or until the pellet is completely dissolved.

3.2 Trypsin Digestion

1. Add 10 μL of 200 mM DTT and 100 μL of TFE to every sample in the analytical run. The reducing agents will break up the disulfide bonds which hold the light and heavy chains of the immunoglobulins together. This will expose the cleavage sites of the light and heavy chains to trypsin.

2. Vortex each tube for 10 s. Cap and incubate at 55 °C for 30 min in a rocking incubator.
3. Cool the mixture down until it reaches room temperature.
4. Add 20 μL of 200 mM IAA for alkylation. Vortex each tube for 10 s. Incubate at room temperature for 1 h in the dark, on an orbital shaker.
5. Transfer 75 μL from each polypropylene tube to a 96 deep-well plate.
6. Once sample transfer is complete, to each well, add:
   (a) 200 μL of water
   (b) 50 μL of 50 mM ammonium bicarbonate
   (c) 50 μL of 1 mg/mL trypsin
7. Cover the plate and vortex for 10–15 s.
8. Sonicate the plate for 1 min.
9. Incubate at 37 °C for at least 8 h up to 24 h (overnight) in a rocking incubator.
10. Add 20 μL of pure formic acid to stop trypsin activity after the overnight incubation.

3.3 LC-MS/MS Set-Up and Quantitation of Infliximab

Peptides (Table 1) are unique to infliximab heavy and light chains, and two transitions are monitored per peptide for added specificity. The primary transition for quantitation is the light chain y6 transition (LC-y6), with the y10 monitored as a qualitative ion. The heavy chain y4 ion (HC-y4) is used as a second quantification ion; the HC-y5 ion was also added as a qualitative ion.

1. Add 5 μL of the 5 μg/mL isotopically labeled standard working solutions (retention time standards) to each well. Vortex the plate for 10 s. These are used to monitor the retention time of each desired peptide (clonotypic peptides from the light and heavy chains of infliximab).
2. Place the plate in the Cohesive LX4 autosampler. Make sure mobile phases A and B are in place, columns are equilibrated, and the chromatography method is set up on your preferred software (*see* **Note 11**).
3. Inject 20 μL of each digest onto a Phenomenex C8 SecurityGuard column (4 × 2.0 mm ID) followed by a Waters XBridge C8 column (3.0 × 30 mm; 3.5 μm) with a flow of 400 μL/min.
4. Separate peptides using a 5.5 min gradient from 95% aqueous (A: water + 0.1% formic acid) to 35% organic (B: acetonitrile + 0.1% formic acid) (Fig. 3).
5. Set instrument Turbo V ion source conditions as IS, 5500; TEM, 600; CAD, 12; CUR, 40; GS1, 35; GS2, 30; and EP, 8. The DP, CE, and CXP values for the SRM transitions were

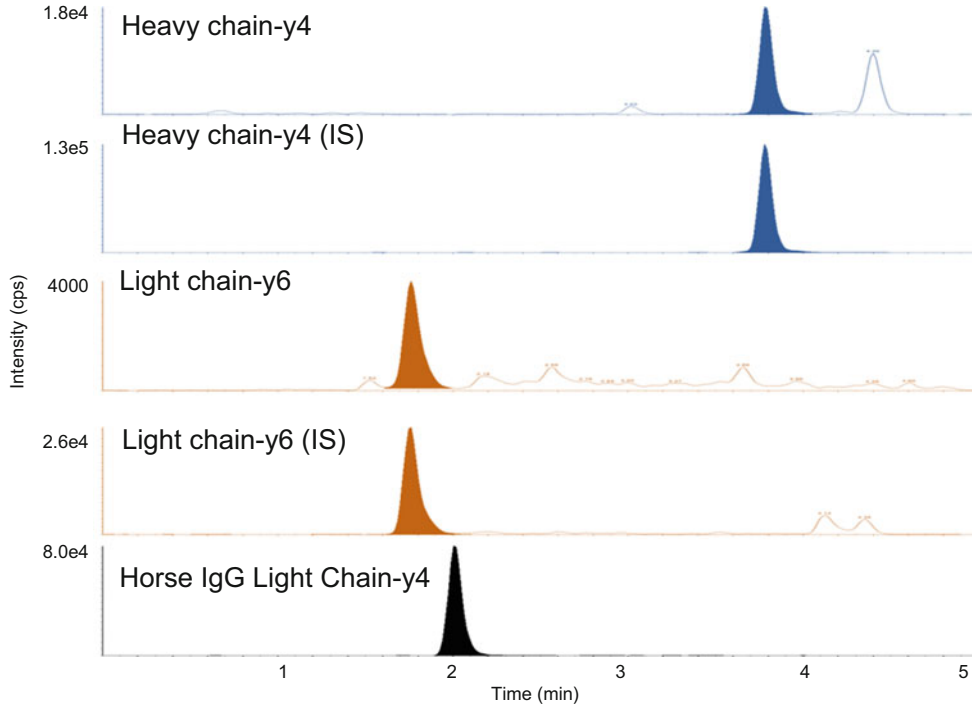

Fig. 3 Chromatograms for heavy chain, light chain, horse IgG, and labeled internal standard peptides transitions. The chromatograms illustrated above show a sample containing 10 μg/mL of infliximab. Horse IgG was added at 50 μg/mL; isotopically labeled internal standards were added at 5 μg/mL

optimized by infusion experiments using isotope-labeled peptides during the test development stage.

6. Generate a 7-point standard curve (1, 2, 5, 10, 20, 50, and 100 μg/mL) using infliximab spiked into pooled human serum standards. Normalize peptide peak areas to horse IgG peptide peak areas (*see* **Note 12**).
7. Quantitate both the light chain y6 and the heavy chain y4 transitions. The two transitions should match within 20% (*see* **Note 13**).
8. Calculated concentrations: Results are automatically calculated by the LC-MS/MS analyst software. Quantitation results are in μg/mL and are read directly from the calibration curve with no corrections unless a sample is diluted. If the sample is diluted (*see* **Note 14**), the technologist should multiply the observed value times the entered dilution factor.
9. Make sure controls used for the assay are within the specified acceptable ranges for the test. We recommend the following Westgard Rules for quality control or CLSI guideline EP05 [16].

4 Notes

1. The saturated ammonium sulfate solution is a critical step in precipitating the immunoglobulins and leaving albumin and other smaller molecular weight proteins in solution. Its preparation is critical for the success of all downstream procedures. The saturated solution should be kept at room temperature for at least 8 h prior to use under stirring. If crystals are not visible after stirring is stopped, make sure to add more ammonium sulfate to the solution under stirring until the crystals become visible.
2. DTT is a reducing agent, which in this protocol will reduce the light chains from the heavy chains of the immunoglobulins and expose the sites of cleavage to trypsin. The reagent should be prepared daily, as its reducing potency is decreased with storage. Preparations stored frozen for more than 24 h significantly decrease the signal intensity of the peptides cleaved by trypsin.
3. IAA serves as an alkylating agent, preventing the reconnection of the disulfide bonds between the immunoglobulin light and heavy chains after they have been reduced by DTT. The reagent is light sensitive and should be kept in the dark until ready to use. The reagent should be prepared fresh daily.
4. Trypsin is a serine protease which cleaves peptide chains at the carboxyl side of amino acids, lysine and arginine, except when either is followed by a proline. The optimal temperature for trypsin activity is at 37 °C, and optimal pH is between 7.8 and 8.7, although there is a degree of hydrolysis at pH closer to 6.5 or 7.0. When adding the trypsin to the prepared sample mixture, monitor the pH in the first few runs to make sure it is within 6.5 and 8.0. If it is not, modifying the amounts of reagents you are adding in each step to reach the desired pH may be helpful for troubleshooting purposes. Trypsin activity is stopped at acid pH below 3.0.
5. During the protocol development stages, we attempted to prepare the standards in buffer or a different matrix other than serum. The results of those experiments were very low or absence of cleavage by trypsin, and therefore, no peptides were generated. This suggests trypsin needs a certain protein ratio to successfully promote cleavage. Therefore, the recommendation is to have standards and controls in serum. The standards should be prepared weekly. We suggest taking out a set of working standards at the beginning of each week and discard any leftover volume at the end of the week. Keep the working standards refrigerated in-between uses.
6. Only reconstitute the drug in water once you are ready to proceed with preparations of calibrators and controls. The

drug is only stable in water for 2 h, according to the package insert.

7. Although not mandatory, to verify the concentration of the working standards, one can use a nephelometric/turbidimetric total IgG assay, available in most clinical laboratories. This can be useful if the working standards prepared do not perform accordingly.
8. Ideally, best laboratory practices recommend that quality control preparations be different from the calibrators used to make the standard curve. However, quality control materials are not readily available for infliximab. Alternatively, residual waste leftover serum from patients can be used as quality control materials if there is enough volume to establish a range of acceptable results and if the sample stability supports such a practice.
9. The tryptic method for quantitation of infliximab described here utilizes a surrogate IS from a different species (horse) since a stable isotope-labeled version of infliximab was not available at time of development and the ones available today are prohibitively expensive. Horse IgG is added to patient serum before immunoglobulin enrichment by ammonium sulfate protein crash to monitor digestion efficiency.
10. The horse IgG works well as an internal standard for this assay since it undergoes trypsin digestion with the sample and accounts for the variation in that process. When analyzing results from quantitation, it is important to observe stability in the peak intensities of the horse IgG transitions. A minimum peak area intensity threshold should be defined, and significant variation (>20%) or trends within an analytical run may be important clues for troubleshooting.
11. Preventive maintenance and rigorous cleaning are very important for instruments used for proteomics assays. The test method shown here is considered relatively "dirty," since samples do not undergo significant extraction or purification. Therefore, an abundant quantity of peptides and small proteins is being injected onto the chromatographic column and the ionization source. It is important to divert non-desired chromatographic portions to waste to save the instrument and minimize interferences. In addition, we suggest a stringent monthly cleaning schedule to prevent build-up.
12. Acceptable standard values should read within 10% of expected target value. The correlation coefficient (R^2) of the curve should be greater than 0.99 for acceptance. Outlying standards may be deleted to improve the observed standard concentrations, R^2 value, and controls. No more than two nonconsecutive standards may be deleted from a particular curve. If the

100 μg/mL standard is deleted, all patient samples with results >50 μg/mL should be repeated. If the 1 μg/mL standard is deleted, concentrations <2 μg/mL cannot be reported.

13. Quantitation from the LC-y6 and HC-y4 ions should match within 20%. Good agreement between quantitation from different transitions is additional evidence of complete digestion by trypsin. Due to the lack of a stable isotopically labeled internal standard for the entire protein, our experience has shown that when the quantitation from the two peptides (HC-y4 and LC-y6) differs by more than 20%, the LC-y6 is the more robust transition to be used for quantitation, since it elutes closer to the retention time of the horse IgG internal standard. When troubleshooting, the HC-y4 transition may show discrepant results in the presence of polyclonal hypergammaglobulinemia in patient samples. In addition, disagreement between HC-y4 and LC-y6 may indicate contamination of the instrument, usually occurring at the ionization source or first quadrupole. Rigorous maintenance is required when that happens.
14. If sample dilutions are required, the dilution should be prepared with normal (infliximab-free) human serum.

Acknowledgments

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Chapter 10

Quantification of Methotrexate in Human Serum and Plasma by Liquid Chromatography Tandem Mass Spectrometry

Gabrielle N. Winston-McPherson, Michael Schmeling, and Andrew N. Hoofnagle

Abstract

Mass spectrometry (MS) is a highly specific and sensitive technique that is used for the detection of many different analytes with diverse chemical characteristics. It has been adopted by clinical laboratories for the quantification of small molecules and, by extension, has been widely used for therapeutic drug monitoring. It is an attractive alternative to immunoassay methods, because it is not subject to the same interferences. A limitation of MS (relative to immunoassays) is the turnaround time. However, this can be addressed by workflow parallelization with other assays. Herein we describe a tandem LC-MS/MS method for the detection and quantification of methotrexate in human plasma with a lower limit of quantification of 0.01 μM and within-assay and between-assay coefficients of variation of less than 15%. This method lacks interference from high-abundance metabolites and utilizes kindred chromatography to improve turnaround time in the therapeutic drug monitoring laboratory.

Key words Methotrexate, Therapeutic drug monitoring, LC-MS/MS

1 Introduction

Mass spectrometry has gained prominence in the clinical laboratory over the last 30 years and is now being used in all areas of clinical diagnostics [1, 2]. The technique lends itself well to the detection and quantification of small molecules that have been traditionally measured using immunoassays. An advantage of mass spectrometry, over immunoassay methods, is the improved specificity [3]. This characteristic is important for assays used in therapeutic drug monitoring, as inaccurate measurements of concentration can be deleterious [4]. In the case of methotrexate, immunoassays suffer from cross-reactivity with methotrexate metabolites, 7-hydroxymethotrexate (7-OHMTX), and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), leading to falsely elevated measurements [5]. Moreover, the availability of methotrexate

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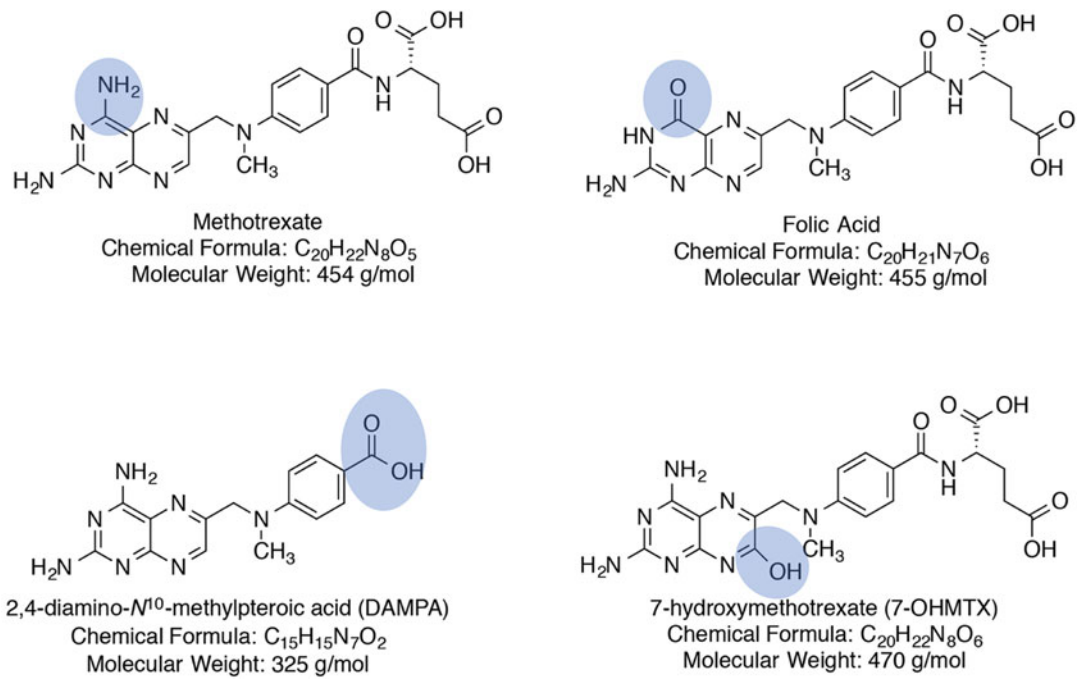

Fig. 1 The structure of folate, methotrexate, and methotrexate metabolites. Key structural differences are highlighted

immunoassays is limited as a widely used platform, the Abbott DX/FLX, was discontinued.

Methotrexate is a toxic folic acid analog that is used clinically as both an immunosuppressant and a chemotherapeutic agent (Fig. 1). It functions by inhibiting essential cellular mechanisms including DNA synthesis and DNA repair [6]. As an immunosuppressant, methotrexate is given at relatively low doses over long periods of time as a treatment for disorders like psoriasis and Crohn's disease. It is given in higher doses, when used as a chemotherapeutic agent, requiring close monitoring and possible rescue with leucovorin [7]. Glucarpidase is another agent used for methotrexate rescue that functions by aiding in the metabolism of methotrexate to DAMPA [8, 9].

Therapeutic drug monitoring of methotrexate is necessary for patients due to possible hepatic toxicity and nephrotoxicity [10]. Additionally, therapeutic drug monitoring is important as there is large pharmacokinetic variability between patients, making proper dosing highly individualized [11, 12]. Mass spectrometry methods for measuring methotrexate have been reported and shown improved performance characteristics over immunoassays [13–16]. It is an attractive alternative to immunoassay methods because it is not subject to the same interferences [17]. One limitation of LC-MS/MS can be delayed turnaround time. However, this can be addressed by utilizing the same chromatographic mobile

phase and solid phase as other assays, with different gradients as needed. This kindred chromatographic design can be applied to different analytes and allows multiple assay workflows to be run in parallel with one another and in series on the LC-MS/MS system, as we have demonstrated previously [18]. For example, the chromatographic column and mobile phases that are used in this procedure are now also used in our laboratory to quantify other therapeutic drugs and metabolites, including sirolimus, cyclosporine, tacrolimus, voriconazole, posaconazole, and teriflunomide, which saves the time it normally takes to change and purge mobile phases and to change and equilibrate columns.

We have developed an LC-MS/MS method for the quantification of methotrexate, after extraction from plasma [18]. The lower limit of quantification is 0.01 μM. This method is not susceptible to analytical interference from methotrexate metabolite 7-OHMTX (MW 470 g/mol) which has a molecular weight difference of only 16 g/mol from methotrexate (Fig. 1). Theoretically, 7-OHMTX could fragment to give a *m/z* ratio the same as methotrexate leading to a positive interference in MS/MS methods. Moreover, this assay is not susceptible to interference by DAMPA (MW 325 g/mol), which has been shown to cause false elevations of methotrexate in immunoassays methods particularly after glucarpidase treatment [4]. In addition, kindred chromatography across therapeutic drug monitoring assays improves turnaround time.

2 Materials

2.1 Equipment and Supplies

1. Waters 2795 XE Alliance HT HPLC system
2. Micromass Quattro Micro™ API tandem mass spectrometer
3. Ascentis Express C18 column, 2.0 cm × 2.1 mm, 2.7 μm
4. Nitrogen generator
5. Multi-tube vortexer
6. Microcentrifuge
7. Adjustable pipettes and appropriate tips
8. Glass vials w/ screw neck
9. Screw cap 12 × 32 PTFE/silicon septa
10. 96-well collection plates
11. Pall adapter collar for centrifugation
12. Nova-Pak C-18 2.1 × 10 mm cartridge, waters
13. Sentry 2.1 mm guard holder, waters
14. Sealing tape pad (3 M)
15. Cap Mat for AcroPrep
16. Defibrinated plasma, UTAK

2.2 Reagents

1. 1 M ammonium acetate: Weigh 38.54 g of crystalline ammonium acetate and transfer to a 1 L graduated cylinder containing 200 mL of water. Add water (~300 mL) to bring the final volume to 500 mL (*see* **Note 1**).
2. 0.1 M NaOH: To a volumetric 100 mL flask, add approximately 70 mL of water. Add 0.4 g of NaOH pellets. Swirl gently to dissolve. Fill to volume with water and mix (*see* **Note 2**).
3. 1 mg/mL methotrexate stock standard: Weigh 5.0 mg of methotrexate and transfer to a bottle containing 5 mL of 0.1 M NaOH. Swirl to mix and aliquot 1 mL into 1.6 mL tubes (*see* **Note 3**).
4. 0.1 mg/mL methotrexate-d_3 stock standard: Weigh 0.5 mg of methotrexate-d_3 and transfer to a bottle containing 5 mL of 0.1 M NaOH. Swirl to mix and aliquot 1 mL into 1.6 mL tubes (*see* **Note 3**).
5. Methotrexate and methotrexate-d_3 tuning solution: Into a 20 mL volumetric flask, add 0.1 mL of 1.0 mg/mL methotrexate stock standard. To the same flask, add 1.0 mL of methotrexate-d_3 stock standard (0.1 mg/mL). Add methanol to a final volume of 20 mL. Swirl to mix (*see* **Note 3**).
6. Precipitation reagent containing internal standard: 0.05 μM methotrexate-d_3 in 1:1 methanol/acetonitrile. In a 25 mL vial, mix 10 mL of HPLC grade acetonitrile and 10 mL of methanol. Add 6 μL of 0.1 mg/mL methotrexate-d_3 standard solution (*see* **Note 4**).
7. Mobile phase A: 2 mM ammonium acetate/0.1% formic acid in water. Measure 1 L of water using a graduated cylinder and add to a 1 L Erlenmeyer flask. Add 2 mL of 1 M ammonium acetate to flask. Add 1.14 mL of formic acid (88%) to flask and swirl to mix (*see* **Note 5**).
8. Mobile phase B: 2 mM ammonium acetate/0.1% formic acid in methanol. Measure 1 L of methanol using a graduated cylinder and add to a 1 L Erlenmeyer flask. Add 2 mL of 1 M ammonium acetate to flask. Add 1.14 mL of formic acid (88%) to flask and swirl to mix (*see* **Note 5**).

2.3 Standards

1. Working standard 1: 10 μM methotrexate. Measure 200 mL of defibrinated plasma using a graduated cylinder and transfer to a bottle. Add 0.91 mL of 1.0 mg/mL methotrexate stock standard. Mix well.
2. Working standard 2: 5 μM methotrexate. Measure 100 mL of defibrinated plasma using a graduated cylinder and transfer to a bottle. Add 100 mL of working standard 1 (10 μM methotrexate). Mix well.

3. Working standard 3: 1 μM methotrexate. Measure 180 mL of defibrinated plasma using a graduated cylinder and transfer to a bottle. Add 20 mL of working standard 1 (10 μM methotrexate). Mix well.
4. Working standard 4: 0.5 μM methotrexate. Measure 100 mL of defibrinated plasma using a graduated cylinder and transfer to a bottle. Add 100 mL of working standard 3 (1.0 μM methotrexate). Mix well.
5. Working standard 5: 0.05 μM methotrexate. Measure 180 mL of defibrinated plasma using a graduated cylinder and transfer to a bottle. Add 20 mL of working standard 4 (0.5 μM methotrexate). Mix well.
6. Working standard 6: 0.01 μM methotrexate. Measure 160 mL of defibrinated plasma using a graduated cylinder and transfer to a bottle. Add 40 mL of working standard 5 (0.01 μM methotrexate). Mix well.
7. Methotrexate low control: UTAK level 2, target value 0.075 μmol/L methotrexate. Reconstitute each vial with 5.0 mL of reagent grade water, using a volumetric pipet. Recap and let it sit for 10–15 min. Swirl gently until a homogeneous mixture is attained. Aliquot 1.0 mL into 1.5 mL polypropylene tubes and store at 4 °C. Stable for 25 days at 4 °C.
8. Methotrexate high control: UTAK level 4, target value 0.75 μmol/L methotrexate. Reconstitute each vial with 5.0 mL of reagent grade water, using a volumetric pipet. Recap and let it sit for 10–15 min. Swirl gently until a homogeneous mixture is attained. Aliquot 1.0 mL into 1.5 mL polypropylene tubes and store at 4 °C. Stable for 25 days at 4 °C.

3 Methods

3.1 Extraction

1. Bring samples and supplies to room temp. Mix all standards, controls, blanks, and samples well before pipetting.
2. Label a 1.6 mL microcentrifuge tube for each blank, standard, control, and sample. The suggested sample order is given below (*see* **Note 6**):
   (a) Blank
   (b) Standard 6
   (c) Standard 5
   (d) Standard 4
   (e) Standard 3
   (f) Standard 2
   (g) Standard 1
   (h) Blank

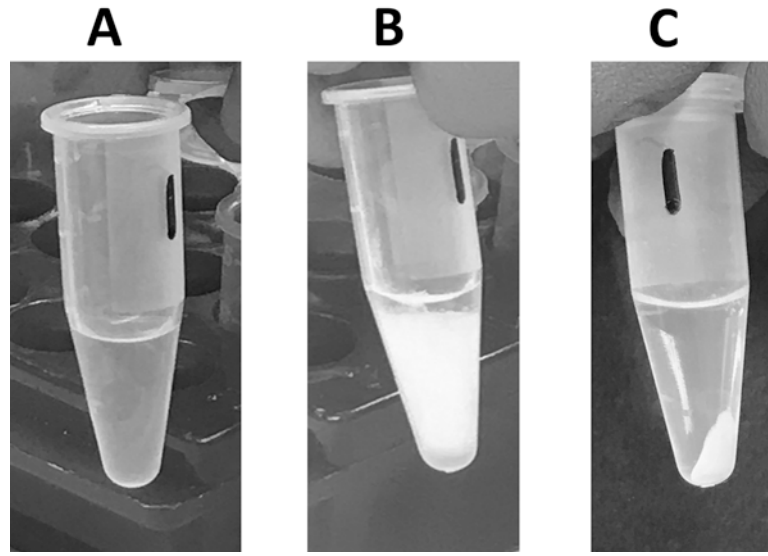

Fig. 2 Protein precipitation with organic solvent. The reaction mixture is shown prior to addition of sample (**a**), after addition of sample and mixing (**b**), and after centrifugation (**c**)

(i) Control low
(j) Control medium
(k) Control high
(l) Experimental/patient specimens

3. Add 400 µL of methotrexate-d_3 precipitation solution to each labeled tube (Fig. 2a).
4. Add 100 µL of blanks, standards, controls, and samples, to the appropriately labeled tube making sure to vortex each sample before addition. After addition, a copious amount of white precipitate should be visible in each sample (Fig. 2b).
5. Place the tubes into the micro-tube vortexer.
6. Cap the tubes, switch the mixing speed knob to the maximum setting, and mix the samples for 5 min (*see* **Notes 7** and **8**).
7. Centrifuge for 10 min at 15,600 × *g* (Fig. 2c).
8. Pipette transfer at least 250 µL of each supernatant into a corresponding 96-well collection plate (*see* **Notes 9** and **10**).
9. Apply 3 M tape (plate seals) on the top of the collection plate to seal.

3.2 LC-MS/MS Analysis

1. Injection volume: 20 µL.
2. Analytical column: Ascentis Express C18 column, 2.0 cm × 2.1 mm, 2.7 µm.
3. Column temperature: 55° C.

Table 1
Chromatographic program for the assay

Time (min)	Flow (min/mL)	Mobile phase A (%)	Mobile phase B (%)
0	0.6	90	10
0.6	0.6	0	100
1.2	0.6	90	10
1.8	End	N/A	N/A

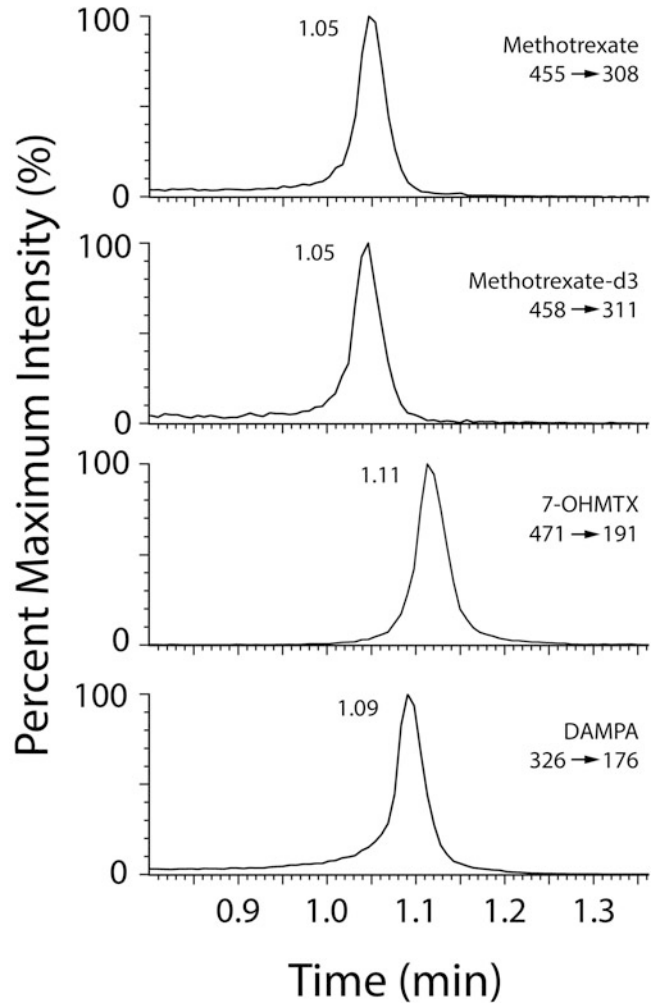

Fig. 3 Liquid chromatography tandem mass spectrometry of the method

4. Solvent elution program: *see* Table 1.
5. Expected retention times: Methotrexate and methotrexate-d_3: 0.95–1.25 min (Fig. 3) (*see* **Notes 11** and **12**).

6. Transitions monitored: Methotrexate *m/z* 455 →308; methotrexate-d_3 *m/z* 458 → 311 (*see* **Note 13**).
7. Instrument parameters are capillary voltage (1 kV), desolvation temperature (400 °C), desolvation gas (nitrogen), collision gas (argon), collision gas pressure (9 psi), cone voltage (30 V), collision energy (20 V), and dwell times (200 ms per channel) (*see* **Note 14**).

4 Notes

1. Once prepared the ammonium acetate reagent is stable for 6 months when stored refrigerated (4–8 °C).
2. The sodium hydroxide reagent is stable at ambient temperature for 1 year after preparation.
3. Working standards made from methotrexate and d_3-methotrexate are stable for 1 year when stored frozen (−20 °C). The tuning solution is stable frozen (−20 °C) for 2 years.
4. The precipitation reagent must be made fresh daily. Observations have shown that the internal standard is unstable in solution. The mixture of methanol and acetonitrile may be prepared several minutes before use, but the internal standard should be added right before the precipitation reagent is to be used (within 30 s to 1 min). Mix the solution well prior to pipetting to ensure thorough incorporation of the internal standard.
5. Mobile phase can be stored at ambient temperature and is stable for 1 month.
6. It is very important to prepare the plate in such a fashion as to minimize potential carryover. This assay has demonstrated carryover at a level of >26 μM methotrexate. Carryover can be minimized by pipetting lower concentration standard prior to high concentration standards. Because the first samples from patients are generally much higher in concentration than subsequent samples from the same patients, they can be pipetted at the end of the batch to avoid carryover in a similar fashion.
7. It is important to secure the tubes in the micro-tube vortexer. Our laboratory uses a cut piece of cardboard that is secured over the tops of all tubes and fixed in place using scotch tape.
8. After mixing, the precipitate may be stuck in the bottom of the tube or on the walls. It is important to dislodge any precipitate that has been collected in the tube and resuspend the mixture prior to centrifugation. This can be achieved by scraping each

tube one time on a tube rack to fully suspend all precipitate, flicking the samples 1–2 times or briefly vortexing.

9. The supernatant can also be poured carefully into a 96-well collection plate. While pipetting is more accurate, pouring the sample is quicker and not susceptible to dripping due to the low surface tension of the supernatant.
10. Place the 96-well collection plate on a dark background prior to transfer of the supernatant. Our laboratory uses a dark colored, laminated, piece of cardstock. Visualization of transfer is much easier when the plate is rested on a dark background.
11. Retention times vary from column to column. Expected retention times are generated as the mean ± SD during development, but observed retention times for patient samples are generally within 0.02 min of the standards for that batch.
12. To evaluate susceptibility to interference from DAMPA or 7-OHMTX, 2 μM 7-OHMTX and DAMPA were added to a residual patient plasma specimen that contained 0.77 μM methotrexate. The sample was extracted per the standard protocol and analyzed using the same chromatographic program. For this experiment, transitions for DAMPA (m/z 326 →176) and 7-OHMTX (m/z 471 →491) were added. Unique retention times were observed for DAMPA (1.09 min) and 7-OHMTX (1.11 min) as compared to methotrexate and methotrexate-d_3 (Fig. 3). The measured concentration of endogenous methotrexate was unchanged from the amount measured prior to addition of 7-OHMTX and DAMPA.
13. Several accreditors and CLSI recommend the use of confirmatory ion transitions in LC-MS/MS assays as best practice in demonstrating the specificity of the assay in each sample. Older, less sensitive instruments, such as the instrument used in this assay, require longer dwell times to achieve the sensitivity that is needed for useful clinical assays. The inclusion of confirmatory ion transitions on these less sensitive instruments decreases the number of peaks across the chromatographic curve, which leads to higher imprecision of the assay. As a result, for therapeutic drug monitoring assays in which the analyte is expected to be present, we rely on the peak shape and retention time of the unlabeled analyte and its labeled internal standard to confirm the specificity of the assay. On newer more sensitive instruments, confirmatory transitions could be added.
14. Please note that these instrument parameters will need to be optimized for different systems.

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Chapter 11

Simultaneous Determination of Tacrolimus and Cyclosporine A in Whole Blood by Ultrafast LC-MS/MS

Matthew W. Bjergum, Paul J. Jannetto, and Loralie J. Langman

Abstract

Numerous methods for the measurement of tacrolimus and cyclosporine A involving traditional liquid chromatography tandem mass spectrometry (LC-MS/MS) have previously been described. The majority of these methods use solid-phase extraction, liquid-liquid extraction, or protein precipitation extraction with instrument run times greater than 15 s per sample. Continued demands in clinical labs for greater efficiency and throughput have put increased stress on traditional technologies such as high-performance liquid chromatography-ultraviolet detection (HPLC-UV) and traditional LC-MS/MS. As an improvement to the existing methods, we describe a sensitive ultrafast LC-MS/MS with run times of less than 15 s per sample.

Key words Prograf, FK506, Neoral, CSA, Gengraf, Mass spectrometry, Rapid fire

1 Introduction

The use of cyclosporine and tacrolimus has led to major advances in the field of transplantation, with excellent short-term outcome concerning prevention of rejection. Patient and graft survival rates have improved secondary to lower incidence of acute rejection episodes and severe infectious complications [1]. However, the toxicity of these drugs is the Achilles' heel of current immunosuppressive regimens. Therapeutic drug monitoring of immunosuppressant drugs that have a narrow therapeutic index is an increasingly useful tool for minimizing toxicity while maximizing prevention of graft loss and organ rejection. Patients treated with calcineurin inhibitors (either cyclosporine or tacrolimus) are at high risk of developing renal injury [2]. Renal effects include tubular dysfunction and rarely hemolytic uremic syndrome that can lead to acute graft loss [3]. Attention must be paid to drug dose, other side effects, and drug interactions to minimize toxicity and maximize efficacy of these drugs. Tacrolimus is a macrolide antibiotic derived from the fungus *Streptomyces tsukubaensis*. Like cyclosporine,

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tacrolimus inhibits calcineurin to suppress T cells. Tacrolimus is metabolized by cytochrome P450 (CYP) 3A4; thus, its concentrations are affected by drugs which inhibit (calcium channel blockers, antifungal agents, some antibiotics, grapefruit juice) or induce (anticonvulsants, rifampin) this enzyme [4].

Since 90% of tacrolimus is in the cellular components of the blood, especially erythrocytes, the whole blood is the preferred specimen for analysis of trough concentrations. Target steady-state concentrations vary depending on clinical protocol, the presence or risk of rejection, time from transplant, type of allograft, concomitant immunosuppression, and side effects (mainly nephrotoxicity). Optimal trough blood concentrations are generally between 5.0 and 15.0 ng/mL. Higher levels are often sought immediately after transplant, but as organ function stabilizes at about 4 weeks from transplant, doses are generally reduced in stable patients for most solid organs [5].

Cyclosporine is a lipophilic polypeptide used to prevent rejection after solid organ transplantation; it suppresses T cell activation by inhibiting calcineurin to decrease IL-2 production. There is substantial inter-patient variability in absorption, half-life, and other pharmacokinetic parameters. Cyclosporine is extensively metabolized by CYP3A4 to at least 30 less active metabolites, many of which are detected on immunoassays [6].

With 80% of cyclosporine sequestered in erythrocytes, the whole blood is the preferred specimen for analysis. Dose is adjusted initially (up to 2 months posttransplant) to maintain concentrations generally between 150 and 400 ng/mL. After the first two postoperative months, the target range is generally lower, between 75 and 300 ng/mL [7].

Cyclosporine A and tacrolimus are each extracted from the whole blood using osmotic shock lysis followed by protein precipitation using methanol and zinc sulfate heptahydrate. The precipitation solution also contains cyclosporine A-$^{13}C_2$,d_4 and ascomycin as internal standards. Samples are vortexed and centrifuged, and the supernatant is analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

2 Materials

All reagents are of HPLC or analytical grade.

2.1 Prepared Reagents

1. Mobile phase 1: 10 mM ammonium acetate, 0.1% formic acid, and 0.01% trifluroacetic acid (TFA) prepared in water. To a 2 L volumetric flask, add 2000 mL of type I water. While stirring, add 1.54 g of ammonium acetate, 2 mL of formic acid, and 180 μL of TFA.

2. Mobile phase 2: 50:50 methanol/water. Add 1 L of methanol to a 2 L volumetric flask. Fill to volume with water and mix well.
3. Mobile phase 3: 10 mM ammonium acetate, 0.1% formic acid, 0.01% TFA, and 10% acetonitrile prepared in methanol. To a 2 L volumetric flask, add 200 mL of acetonitrile. While stirring, add 1.54 g of ammonium acetate, 2 mL of formic acid, 180 µL of TFA, and 1800 mL of methanol.
4. Tacrolimus stock II standard, 10 µg/mL tacrolimus: Add 100 µL of 1.0 mg/mL tacrolimus reference standard to a 10 mL volumetric flask, bring to volume with methanol, and mix well. Stable up to 2 years stored at −20 °C in screw-cap amber vials with rubber/Teflon septa.
5. Cyclosporine A-$^{13}C_2$,d_4 stock II standard, 100 µg/mL cyclosporine A-$^{13}C_2$,d_4: Add 5.0 mg of cyclosporine A-$^{13}C_2$,d_4 to a 50 mL volumetric flask, bring to volume with methanol, and mix well. Stable up to 2 years stored at −20 °C in screw-cap amber vials with rubber/Teflon septa.
6. Ascomycin stock II standard, 100 µg/mL ascomycin: Add 5.0 mg of ascomycin to a 50 mL volumetric flask, bring to volume with methanol, and mix well. Stable up to 2 years stored at −20 °C in screw-cap amber vials with rubber/Teflon septa.
7. Working Standard 1: 1.0 ng/mL tacrolimus/25 ng/mL cyclosporine A. Add 20 mL of water to a 1000 mL volumetric flask, then add 100 µL of tacrolimus stock II standard and 25 µL of 1.0 mg/mL cyclosporine A reference standard. Bring to volume with bovine whole blood and mix well (*see* **Note 1**). Stable up to 6 months at or below at −60 °C.
8. Working Standard 2: 5.0 ng/mL tacrolimus/150 ng/mL cyclosporine A. Add 20 mL of water to a 1000 mL volumetric flask, then add 500 µL of tacrolimus Stock II standard and 150 µL of 1.0 mg/mL cyclosporine A reference standard. Bring to volume with bovine whole blood and mix well. Stable up to 6 months at or below at −60 °C.
9. Working Standard 3: 10.0 ng/mL tacrolimus/400 ng/mL cyclosporine A. Add 20 mL of water to a 1000 mL volumetric flask, then add 1000 µL of tacrolimus Stock II standard, and 400 µL of 1.0 mg/mL cyclosporine A reference standard. Bring to volume with bovine whole blood and mix well. Stable up to 6 months at or below at −60 °C.
10. Working Standard 4: 20.0 ng/mL tacrolimus/700 ng/mL cyclosporine A. Add 20 mL of water to a 1000 mL volumetric flask, then add 2000 µL of tacrolimus Stock II standard and 700 µL of 1.0 mg/mL cyclosporine A reference standard.

Bring to volume with bovine whole blood and mix well. Stable up to 6 months at or below at −60 °C.

11. Working Standard 5: 40.0 ng/mL tacrolimus/1000 ng/mL cyclosporine A. Add 20 mL of water to a 1000 mL volumetric flask, then add 4000 µL of tacrolimus Stock II standard and 1000 µL of 1.0 mg/mL cyclosporine A reference standard. Bring to volume with bovine whole blood and mix well. Stable up to 6 months at or below at −60 °C.
12. Working Internal Standard: 2.0 ng/mL ascomycin/50 ng/mL cyclosporine A-$^{13}C_2$,d_4. Add 20 mL of water to a 2000 mL volumetric flask, add 56 g of zinc sulfate heptahydrate, and mix until completely dissolved. Add 1800 µL of methanol, 1000 µL of 100 µg/mL Cyclosporine A-$^{13}C_2$,d_4, and 50 µL of 100 µg/mL ascomycin; mix well. Stable up to 14 days at or below at −10 °C.
13. Quality control 1: 5.0 ng/mL tacrolimus/100 ng/mL cyclosporine A, in drug-free whole blood. Obtained commercially.
14. Quality control 2: 15.0 ng/mL tacrolimus/300 ng/mL cyclosporine A, in drug-free whole blood. Obtained commercially.
15. Quality control 3: 30.0 ng/mL tacrolimus/500 ng/mL cyclosporine A, in drug-free whole blood. Obtained commercially.

2.2 Supplies and Analytical Equipment

1. Agilent RapidFire 365.
2. Triple quadrupole mass spectrometer using electrospray ionization source. e.g., Agilent Model 6495 or similar.
3. Centrifuge with deep well plate holders.
4. SPE cartridge: Agilent RapidFire-C_{18}.
5. 96 deep-well plates. 1.5 mL.
6. 96 square-well plates. 2.0 mL.

3 Methods

3.1 Preparation of Working Standards, Controls, and Unknown Samples (Each Run)

1. Prepare working calibration standards, quality controls, and unknown samples for the run: Mix samples for 5 min. Add 200 µL of each working calibration standard, quality control, and unknown sample to its own well of a 96 square-well plate, 2.0 mL.
2. Add 200 µL of water to each sample well (*see* **Note 2**).
3. Vortex plate for 15 s.
4. Add 300 µL of working internal standard to each well (*see* **Note 3**).

5. Vortex and centrifuge for 10 min at 2845 g.
6. Transfer 400 μL of each standard, control, and sample supernatant to a 96 deep-well plate, 1.5 mL.

3.2 Analysis

1. Place the sample extracts in the 96-well plate in the following order (*see* **Note 4**):

Calibration standards, in order of lowest to highest concentration (*see* **Note 5**).

Blank whole blood (carryover) control immediately after highest-concentration calibration standard (*see* **Note 6**).

Quality controls, in order of lowest to highest concentration (*see* **Note 7**).

Unknown samples and additional quality controls.

2. Set RapidFire 365 method to the following parameters:
   (a) Pump flow rates:
   Pump 1, mobile phase 1: 1.5 mL/min.
   Pump 2, mobile phase 2: 1.5 mL/min.
   Pump 3, mobile phase 3: 1.25 mL/min.
   (b) Cycle durations in milliseconds:
   Aspirate state/volume: 600 (*see* **Note 8**).
   Load/wash state: 3000.
   Extra wash state: 2000.
   Elute state: 3500.
   Re-equilibrate state: 500.
   (c) Ion source parameters: gas temp = 150 °C, gas flow = 14 L/min, nebulizer = 20 psi, sheath gas temp = 300 °C, sheath gas flow = 11 L/min, nozzle voltage = 300 V, and capillary voltage = 5000 V.
   (d) iFunnel parameters: high pressure = 120 V and low pressure = 80 V.
   (e) Mass spectrometer parameters: Compound-dependent parameters are detailed in Table 1 (*see* **Note 9**).
3. Figure 1 shows the chromatography of a working standard containing tacrolimus and internal standard (ascomycin).
4. Figure 2 shows the chromatography of a working standard containing cyclosporine A and internal standard.

Table 1
Analytical and detection conditions for tacrolimus, cyclosporine A, and internal standards

Drug	Retention time (Min.)	Precursor ion (*m/z*)	Quantifier product ion (*m/z*)	Quant. collision energy (V)	Qualifier product ion #1 (*m/z*)	Qual. #1 collision energy (V)	Mean ratio qualifier MRM #1
Tacrolimus	0.145	821.8	768.5	17	786.5	13	35
Ascomycin	0.146	809.6	756.6	17	774.6	14	41
Cyclosporine A	0.144	1219.8	1202.6	14	1184.5	30	9
Cyclosporine A- $^{13}C_2$,d_4	0.144	1225.8	1208.6	15	–	–	–

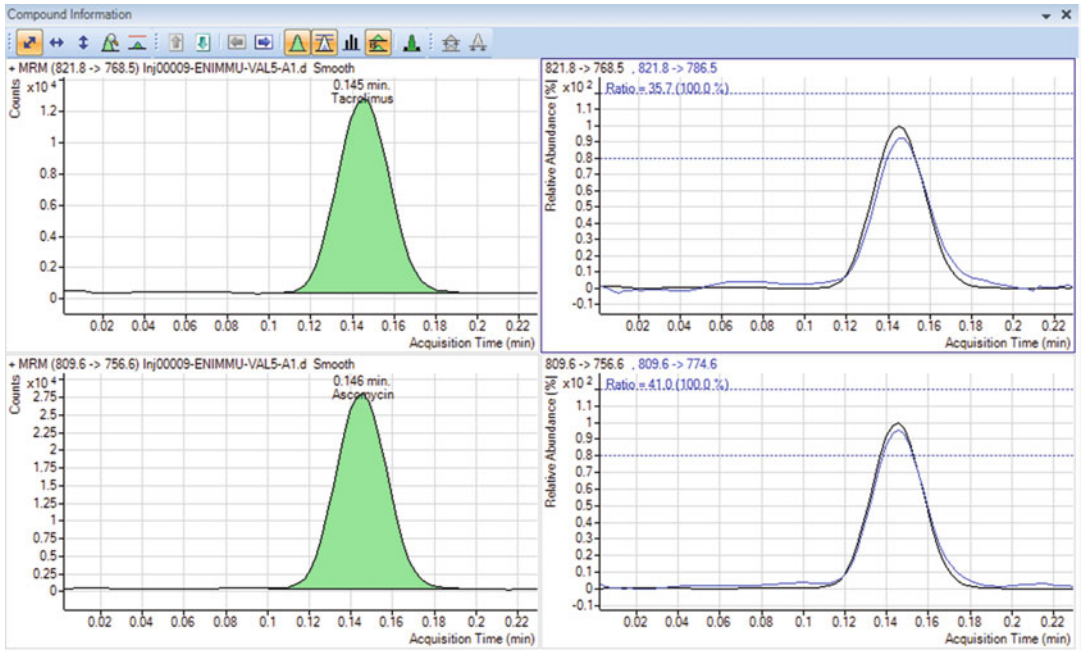

Fig. 1 Chromatography of a working standard containing tacrolimus and internal standard (ascomycin)

4 Notes

1. Use freshly thawed and strained bovine whole blood. Add appropriate volume of stock II standard, mix, and allow to equilibrate for 24 h.
2. Tacrolimus is extensively bound to the FK-binding protein within the red blood cells (RBCs). Osmotic lysis is used to burst the RBCs and thus releasing tacrolimus protein complexes.

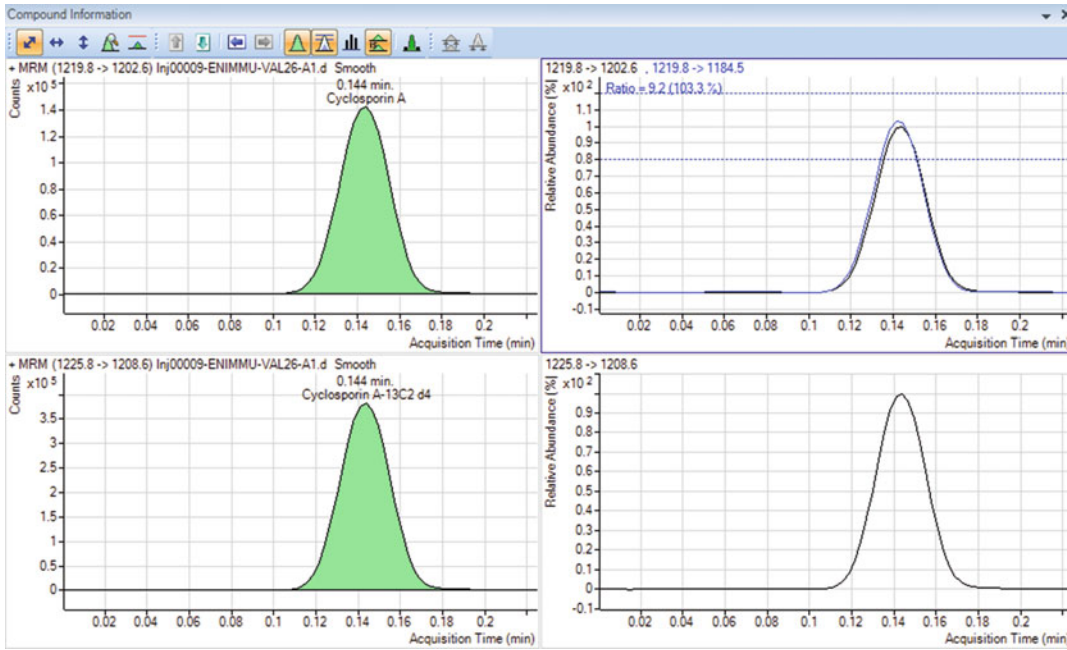

Fig. 2 Chromatography of a working standard containing cyclosporine A and internal standard

3. Zinc sulfate/methanol denatures and dissociates the protein which causes the release of tacrolimus.
4. Sample order is at the discretion of the user; the rationale for this order is as follows. The highest concentration standard is placed last in the calibration curve and is followed by a blank sample to assess any carryover. Quality control samples are interspersed with unknown samples to monitor the success of analysis throughout the run. To ensure at least 10% of each clinical run is comprised of quality controls and calibrators, we run one control after every nine patient samples.
5. Ensure that the curve is drawn correctly and there are no points that are excluded from a good linear or quadratic fit. The recommended R^2 value is greater than 0.990. Cyclosporine standards were calibrated based on a quadratic $1/x^2$ curve fit. Tacrolimus standards were calibrated based on a linear $1/x^2$ curve fit.
6. A peak with the same retention time as tacrolimus or cyclosporine should have a calculated concentration of less than 50% of working standard.
7. Calculated results should fall within two standard deviations of the expected value, exhibit good chromatography, and stable retention times.
8. The instrument aspirates for a specified time rather than specified volume.

9. Quantifier and qualifier MRM (multiple reaction monitoring) transitions are based on a single precursor ion for each drug. Cyclosporine internal standard produces minimal fragment ions that are reliable and reproducible. Mass spectrometry parameters will vary between instruments and must be optimized for the specific LC-MS/MS used.

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Chapter 12

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Method to Quantify Gabapentin and Pregabalin in Urine

Stephen Merrigan and Kamisha L. Johnson-Davis

Abstract

Gabapentin and pregabalin are anticonvulsant drugs that are also utilized for pain management. A mass spectrometry method was developed and validated to quantify gabapentin and pregabalin in urine to support testing for adherence.

Key words Anticonvulsants, Mass spectrometry, LC-MS/MS, Urine, Pain management

1 Introduction

Gabapentin (Neurontin) is an anticonvulsant drug that was approved for use as an adjunctive treatment of partial seizures in both children and adults [1]. Gabapentin is also indicated for the management of postherpetic neuralgia in adults [2, 3]. It is also used as treatment for neuropathic pain following spinal cord injury, post-traumatic stress disorder, poststroke pain syndrome, alcohol withdrawal, migraine therapy, hot flashes associated with prostate cancer treatment, and postoperative pain after cancer surgery [3, 4]. Although gabapentin is structurally similar to gamma-amino butyric acid (GABA), gabapentin does not interact with GABA receptors, nor is it converted to GABA or a GABA agonist [5, 6]. The general mechanism by which gabapentin exerts its anticonvulsant action is unknown; however, the drug binds to the alpha 2-delta subunit of the voltage-gated calcium channels in the CNS to decrease calcium influx, which will reduce neurotransmitter release [4]. Gabapentin is not metabolized in the liver, nor does it induce liver enzymes. The drug circulates relatively unbound in serum, with a protein-bound fraction of about 3% and has a volume of distribution of approximately 58 L [6, 7]. Gabapentin is

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eliminated via the kidneys with an elimination half-life of approximately 5–7 h [5–7]. Impaired renal function can substantially decrease the clearance of gabapentin. The major side effects of the drug include somnolence, dizziness, ataxia, fatigue, and nystagmus. Administration of gabapentin does not influence the pharmacokinetics of conventional anticonvulsant drugs nor are the pharmacokinetics of gabapentin modified by the presence of other anticonvulsant drugs [5].

Pregabalin (Lyrica) is an analog of the inhibitory neurotransmitter GABA, as well as gabapentin. The mechanism of action is not fully elucidated; however, it is known that the drug binds to the alpha2-delta subunit of the voltage-gated calcium channels in the CNS to decrease neurotransmitter release [8]. The drug is utilized for the management of neuropathic pain associated with diabetic peripheral neuropathy, postherpetic neuralgia, fibromyalgia, generalized anxiety disorder, spinal cord injury, and others [2, 9–13]. Pregabalin is also indicated for adjunctive therapy for adult patients with partial-onset seizures [3, 14]. Pregabalin is rapidly absorbed after oral administration; the drug is not highly protein bound; it is not metabolized by the liver but is excreted in the urine, unchanged [15, 16]. The elimination half-life is around 5–7 h., and the volume of distribution is about 0.5 L/kg. Pregabalin side effects include asthenia, dry mouth, constipation, peripheral edema, dizziness, somnolence, ataxia, confusion, and blurred vision [8].

Therapeutic drug monitoring (TDM) of gabapentin and pregabalin for seizure management is not widely employed but is clinically beneficial, especially in patients with poor renal function, since both drugs are excreted via the kidneys. TDM is typically performed on serum/plasma samples in order to assist clinicians to manage drug therapy within the therapeutic range. In the field of pain management, pregabalin and gabapentin can be used in combination with other drugs to treat pain, such as opiates and benzodiazepines [17]. Urine drug testing is widely utilized to assist clinicians to assess patient adherence/compliance to prescribed medications. A mass spectrometry method to quantify gabapentin and pregabalin in urine can be used to support testing for medication adherence/compliance.

2 Materials

2.1 Supplies and Equipment

1. Cellulose acetate filter for plasma preparation, 0.45 μm.
2. Volumetric glassware and pipettes.
3. 10 L container with a spigot or tap.
4. Transfer pipettes with tips.
5. 96 deep-well plate.

6. 96 deep-well plate mat cover.
7. Centrifuges with rotors for 96-well plates and for 250 mL bottles.
8. CTC Pal autosampler with cooled sample tray, Agilent series 1200 HPLC pump, and an AB SCIEX API 4000 mass spectrometer with analyst software 1.5.1. or similar.
9. Phenomenex Kinetex, 2.1 mm × 50 mm 2.6 μm, Biphenyl HPLC column, or similar.
10. AB SCIEX Quantitation Wizard Software.

2.2 Solutions

1. Synthetic urine: Add 100 g of urea, 35 g of sodium chloride, 14 g of potassium phosphate, 5.0 g of creatinine, and 2.75 g of sodium phosphate to a 5 L beaker. Fill to volume with ≥18 MΩ water. Stir to mix. Store at <−60 °C, stable for 1 year.
2. Blank plasma, e.g., expired plasma from a blood bank: Centrifuge plasma at 7826 rcf for 30 min at 4 °C, filter through 0.45 μm cellulose acetate filter to remove particulates. Test plasma to ensure it is negative for gabapentin and pregabalin. Store at <−20 °C, stable 6 months (*see* **Note 1**).
3. Calibrator 1: 0.25 μg/mL gabapentin and pregabalin. Add 12.5 μL each of 1.0 mg/mL gabapentin and pregabalin to a labeled 50 mL volumetric flask . Fill to volume with blank plasma. Add a stir bar and insert the stopper. Stir at room temperature for 1 h or until all reference material is in solution (*see* **Note 2**).
4. Calibrator 2: 0.5 μg/mL gabapentin and pregabalin. Add 25 μL each of 1.0 mg/mL gabapentin and pregabalin to a labeled 50 mL volumetric flask. Fill to volume with blank plasma. Add a stir bar and insert the stopper. Stir at room temperature for 1 h or until all reference material is in solution.
5. Calibrator 3: 2.5 μg/mL gabapentin and pregabalin. Add 125 μL each of 1.0 mg/mL gabapentin and pregabalin to a labeled 50 mL volumetric flask. Fill to volume with blank plasma. Add a stir bar and insert the stopper. Stir at room temperature for 1 h or until all reference material is in solution.
6. Calibrator 4: 5.0 μg/mL gabapentin and pregabalin. Add 250 μL each of 1.0 mg/mL gabapentin and pregabalin to a labeled 50 mL volumetric flask. Fill to volume with blank plasma. Add a stir bar and insert the stopper. Stir at room temperature for 1 h or until all reference material is in solution.
7. Calibrator 5: 25 μg/mL gabapentin and pregabalin. Add 1250 μL each of 1.0 mg/mL gabapentin and pregabalin to a labeled 50 mL volumetric flask. Fill to volume with blank plasma. Add a stir bar and insert the stopper. Stir at room temperature for 1 h or until all reference material is in solution.

8. Calibrator 6: 50 μg/mL gabapentin and pregabalin. Add 2500 μL each of 1.0 mg/mL gabapentin and pregabalin to a labeled 50 mL volumetric flask. Fill to volume with blank plasma. Add a stir bar and insert the stopper. Stir at room temperature for 1 h or until all reference material is in solution.
9. Calibrator 7: Blank. Process 50 mL blank plasma as described above, but do not add gabapentin or pregabalin stock solutions.
10. Quality control 1: 5 μg/mL gabapentin and pregabalin. Add 15 mL synthetic urine to a labeled 25 mL volumetric flask. Add 125 μL each of 1.0 mg/mL gabapentin and pregabalin. Fill to volume with synthetic urine. Add a stir bar and insert a stopper into the flask. Stir the solutions for 1 h at room temperature (*see* **Note 3**).
11. Quality control 2: 15 μg/mL gabapentin and pregabalin. Add 15 mL synthetic urine to a labeled 25 mL volumetric flask. Add 375 μL each of 1.0 mg/mL gabapentin and pregabalin. Fill to volume with synthetic urine. Add a stir bar and insert a stopper into the flask. Stir the solutions for 1 h at room temperature.
12. Internal standard/precipitation solution: Obtain a 10 L container with a spigot or tap. Add 4.0 L of methanol and 4.0 L of acetonitrile to the container. Add 4.8 mL of 0.1 mg/mL gabapentin-d_{10} internal standard and 4.8 mL of 0.1 mg/mL pregabalin-d_6 internal standard using an accurate syringe or pipette. Add a stir bar and mix at room temperature for 1 hour. Aliquot into appropriately labeled 1.0 L bottles (*see* **Note 4**).
13. Mobile phase A/postcrash diluent: 0.1% formic acid in clinical laboratory reagent water**.** Add 1 mL of formic acid to a 1 L beaker; fill to 1 L with $\geq$18 MΩ water. Stir to mix. Store at 23 °C, stable 1 month (*see* **Note 5**).
14. Mobile phase B: 0.1% formic acid in acetonitrile. Add 1 mL formic acid to a 1 L beaker; fill to 1 L with mass spectrometry grade acetonitrile. Store at 23 °C, stable for 3 months.
15. 1:1:1 methanol:acetonitrile:water (v/v). Add 10 mL each of HPLC grade methanol, acetonitrile, and water to a 50 mL glass bottle and cap. Make fresh before use.
16. Prime intermediate solution: Add 10 μL each of 1.0 mg/mL gabapentin and pregabalin stocks to a 5 mL volumetric flask and bring to volume with HPLC grade methanol. Mix well. Store at 4 °C, stable for 3 months.
17. Prime solution: Add 325 μL of the prime intermediate solution to a 25 mL volumetric flask and bring to volume with 1:1:1 methanol:acetonitrile: water. Transfer 1.6 mL of prime solution to autosampler vials and cap. Stable for 1 year at <-60 °C.

3 Methods

3.1 Sample Preparation

1. Pipette 20 µL of each calibrator, quality control, and patient sample into its own well of a 96-well plate (*see* **Note 6**).
2. Add 380 µL internal standard precipitation solution to each well and seal with a plate mat.
3. Mix by a 30 s vortex at 1006 rcf.
4. Centrifuge for 5 min at 3500 rcf.
5. Pipette 380 µL of postcrash diluent (mobile phase A) into each well of a second 96-well plate.
6. Using the 8-channel pipette, pipette 20 µL supernatant from precipitated sample plate into the second plate containing the postcrash diluent with pipette tips immersed in liquid. Rinse tips by drawing diluent into expelled sample tips and re-expelling.
7. Seal the plate with a plate mat cover.

3.2 Analysis

This assay employs electrospray ionization in positive ion mode and multiple reaction monitoring (MRM) of mass transitions.

1. Set autosampler parameters according to Table 1. The injection volume is 10 µL.
2. Set mobile phase gradient according to Table 2. The flow rate is 0.4 mL/min (*see* **Note 7**).
3. Set integrated switching valve parameters according to Table 3.
4. Set mass spectrometer conditions according to Table 4.
5. Run samples. Retention times and MRM transitions are shown in Tables 5 and 6, respectively.
6. Analyze data. The calibration curve is linear with $1/x$ weighting (*see* **Note 8**).

4 Notes

1. The calibrators were made in plasma because this assay was designed to run both urine and plasma matrices. Sample preparation for the two matrices is the same.
2. Calibrators and blank can be transferred in 400 µL aliquots to labeled tubes and capped tightly. Placed upright in a <-60 °C freezer; these solutions are stable for 1 year.
3. Controls can be transferred in 150 µL aliquots to appropriately labeled tubes. Cap tightly. Store at <-60 °C and the solution is stable for 1 year.

Table 1
Autosampler parameters

Loop volume 1 (μL)	20
Injection volume (μL)	10
Airgap volume (μL)	3
Front volume (μL)	5
Rear volume (μL)	5
Filling speed (μL/s)	5
Pull-up delay (ms)	3000
Inject to	LC Vlv1
Injection speed (μL/s)	5
Pre inject delay (ms)	500
Post inject delay (ms)	500
Needle gap valve clean (mm)	3
Valve clean time solvent 1 (s)	3
Valve clean time solvent 2 (s)	3
Post clean time solvent 1 (s)	2

Table 2
Mobile phase gradient

Step	Total time (min)	Flow rate (μL/min)	Mobile phase aqueous (%)	Mobile phase organic (%)
0	0.00	400	95.0	5.0
1	1.50	400	87.0	13.0
2	2.00	400	83.0	17.0
3	2.10	400	5.0	95.0
4	2.90	400	5.0	95.0
5	3.00	400	95.0	5.0
6	3.50	400	95.0	5.0

4. The internal standard precipitation solution will be run with every batch of samples using prepared aliquots. Store at 2–8 °C and the solution is stable for 1 year. Validate the solution prior to placing into use by confirming performance of a calibration curve and accuracy of control values.

Table 3
Integrated valve parameters

Valco valve	Diverter	
Position	Total time (min)	Position
Default position	0	Left
1	1.0	Right
2	2.0	Left

Table 4
Mass spectrometer parameters

Curtain gas (CUR)	50 psi
Ion spray voltage (IS)	2000 V
Temperature (TEM)	500 °c
Ion source gas 1 (GS1)	55 psi
Ion source gas 2 (GS2)	60 psi
Collision gas (CAD)	7
Declustering potential (DP)	50 V
Entrance potential (EP)	10 V

Table 5
Approximate retention times

Drug	Retention time (minutes)
Gabapentin	1.51
Gabapentin-d_{10}	1.46
Pregabalin	1.23
Pregabalin-d_6	1.2

5. Prepare the mobile phases as accurately as possible. Variations may affect retention times.
6. Urine samples in plastic containers are acceptable, refrigerated (4 °C) up to 1 month or frozen (−20 °C) for up to 1 month. Assay procedure is performed at room temperature. If patient samples are frozen, allow samples to thaw before analysis.
7. Allow the column to equilibrate for 20 min before use.
8. Acceptable calibration requires an R^2 value greater than 0.995 and control values within two standard deviations of the

Table 6
MRM transitions

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	Collision energy (V)	Collision exit potential (V)	Dwell (m s)
Gabapentin (quantifier ion)	172.1	137.1	21	27	50
Gabapentin (qualifier ion)	172.1	95.1	31	15	50
Pregabalin (quantifier ion)	160.1	125.2	18	25	50
Pregabalin (qualifier ion)	160.1	83.2	22	15	50
Gabapentin-d_{10} (quantifier ion)	182.1	147.2	21	27	50
Gabapentin-d_{10} (qualifier ion)	182.1	103.5	31	15	50
Pregabalin-d_6 (quantifier ion)	166.1	131.1	18	25	50
Pregabalin-d_6 (qualifier ion)	166.1	89.2	22	15	50

mean. Quantitation is based on peak area ratio of analyte to deuterated internal standard in the sample compared to the calibration curve. Specificity of analysis is confirmed by the respective qualitative mass transition ratio in the sample compared to average transition ratios in the calibration curve ±30%.

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Chapter 13

The Evolving Landscape of Designer Drugs

Sherri L. Kacinko and Donna M. Papsun

Abstract

Since 2008 there has been an onslaught of new drugs in the illicit marketplace. Often referred to as "research chemicals," "designer drugs," or "novel psychoactive substances" (NPS), these substances are used for their pharmacological effects which are often similar to more widely known drugs such as ecstasy or heroin. In some cases users specifically seek out these new chemicals, in other cases they are simply purchasing what they believe to be their normal drug of choice from a dealer, but the product is not what it is purported to be. Implementation of national and international systems to monitor the appearance of new compounds enables laboratories to be prepared with validated tests to detect them in biological specimens. The most common classes of NPS are synthetic cannabinoids, novel opioids, novel benzodiazepines, stimulants, and hallucinogens. Within these groups the compounds may be drugs that were originally synthesized for research purposes during the pursuit of new therapeutic agents such as the synthetic cannabinoid JWH-018 and the designer opioid U47700. Others like etizolam are compounds used in other countries but not commonly seen in the USA. Some are drugs synthesized specifically to circumvent legal controls. In all cases, these compounds present a unique challenge to forensic toxicology laboratories which must quickly develop and validate analytical methods for the identification and quantification in biological matrices.

This chapter is a condensed and updated version of an article originally published in Clinical and Forensic Toxicology News.

Key words Research chemicals, Novel psychoactive substances, Synthetic drugs, Synthetic cannabinoids, Analogs

1 History of Novel Psychoactive Substances [1]

Designer drugs, research chemicals, novel psychoactive substances (NPS), and "legal highs" are terms used to describe drugs designed specifically to skirt legal controls. Although this movement picked up steam in the new millennium, the search for new recreational substances, either through repurposing pharmaceutical research or modifying existing drugs of abuse, is not new.

The second International Opium Convention, effective in 1928, specifically banned morphine and heroin [2]. In response, chemists began synthesizing esters of morphine with similar

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structures and effects to heroin such as benzoylmorphine and acetylpropionylmorphine, which were considered legal alternatives to morphine and heroin.

Dr. Albert Hofmann, a chemist at Sandoz Laboratories in Basel, Switzerland, was investigating potential medical uses of ergot alkaloid derivatives when he synthesized lysergic acid diethylamide (LSD) in 1938. The psychedelic properties of LSD were discovered when Dr. Hofmann accidently ingested the drug, and in 1947 Sandoz Laboratories introduced it as a medication for the treatment of psychiatric disorders [3]. LSD continued to be a popular drug for psychiatric research and recreational use and was eventually controlled by the DEA in 1968. Shortly thereafter the synthesis of ALD-25 or 1-acetyl-LSD led to the first prosecution of a drug analogue.

In 1965, Dr. Alexander Shulgin obtained a drug enforcement agency (DEA) license and began synthesizing chemicals in a backyard laboratory, thus launching the modern era of designer drugs. Dr. Shulgin developed a protocol for evaluating new drugs which included initial testing in animals before human experiments began. The protocol included details on determining an effective dose by administration of increasing doses and use of a rating scale. The subjects of these experiments were friends and acquaintances of Dr. Shulgin and his wife. Comprehensive narratives on these experiences were published in two books, *Phenethylamines I have Known and Loved* (PIHKAL, 1991) and *Tryptamines I have Known and Loved* (TIHKAL, 1997). These two books are often considered to be "cookbooks" used by manufacturers looking to introduce these drugs to a new generation of users.

While Dr. Shulgin was evaluating stimulant and hallucinogenic drugs in California, Dr. John W. Huffman was at Clemson University studying the cannabinoid receptor system to gain more understanding of their role in diseases such as multiple sclerosis. As part of his research, Dr. Huffman and his team synthesized more than 400 compounds which have varying degrees of binding affinity and activity at cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). CB1 is the receptor which is responsible for the desired effects of delta-9-tetrahydrocannabinol (THC), the primary active component of marijuana, and CB2 is primarily a peripheral receptor involved in immune system modulation. Dr. Huffman was searching for compounds with affinity for and activity at the CB2 receptor, but over two decades later, experimental drug users "rediscovered" Dr. Huffman's work and the potential in CB1 receptor agonists.

2 Monitoring NPS

In 2007, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) updated its guidelines for the early warning system (EWS) [4]. The purpose of this update was to expand the scope from new synthetic drugs to all new psychoactive substances. Under these guidelines a steady increase in the number and scope of new compounds has been reported every year since 2008. Not only has the number of monitored compounds increased; the types are also changing. In addition to synthetic cannabinoids, opioids and benzodiazepines have gained popularity in recent years.

In the USA, the National Drug Early Warning System (NDEWS) was created in 2014 as a cooperative agreement between the National Institute on Drug Abuse and the University of Maryland [5]. NDEWS collects information from a variety of sources including localized early warning systems to provide a national warehouse for information on drug trends and emerging novel psychoactive substances.

3 Classes of NPS

3.1 Synthetic Cannabinoids

The first incidence of synthetic cannabinoids being detected in the USA occurred in late 2008 when US Customs and Border Patrol verified the presence of HU-210 in packets of an herbal incense product called "spice." HU-210 was already a controlled substance, but shortly thereafter another uncontrolled drug, JWH-018, was detected in similar products. This compound was one of the 400 substances synthesized by Dr. Huffman. Since early 2009 the components of these herbal incenses have changed rapidly. As soon as the DEA adds a substance to the list of scheduled compounds, chemists make slight modifications to the structure to create a compound that is not listed.

A thorough review on the pharmacology and toxicology of synthetic cannabinoids was published in 2014 and updated, along with other classes of synthetic cannabinoids, in 2017 [6, 7]. Recreational synthetic cannabinoids target the CB1 receptor though many also bind to and have an effect at the CB2 receptor. It is possible that CB2 receptor activity modulates some of the undesirable effects seen with the pure CB1 receptor agonists. The most common in vivo test for cannabinoids is the mouse tetrad test which evaluates the effect of a compound on several different mouse behaviors. This series of tests determines the dose of drug required to change specific behaviors by 50% (ED50). By comparing the ED50 of one drug with the ED50 of another drug, the potency can be estimated. Unfortunately, this information is only available for a handful of synthetic cannabinoids being used; the mouse tetrad has not been performed on the vast majority of substances.

General adverse effects associated with synthetic cannabinoid use include tachycardia, hypertension, agitation, hallucinations, nausea, vomiting, tremors, seizures, anxiety, and paranoia. The type and degree of effect vary significantly between different compounds, and some compounds such as XLR-11 have been associated with specific health effects. Following a cluster of exposures to XLR-11, researchers noted that there appeared to be an association between exposure to this substance and acute kidney injury [8]. Furthermore, synthetic cannabinoids have been indicated by pathologists as a cause of death [7].

3.2 Opioids

Novel opioid compounds have a longer history than synthetic cannabinoids, but after a long period of inactivity with respect to new compounds, acetyl fentanyl burst onto the recreational drug scene in 2013, and then in late 2015, numerous other opioid-related compounds began to appear. These primarily consist of substances synthesized by pharmaceutical companies looking for new analgesic drugs for pain treatment which were abandoned at some point in the development process. These represent a chemically diverse class of substances, most of which do not have structural similarity to opiates.

There is evidence that these drugs are both sought after by drug users for recreational use and by drug manufacturers as adulterants to more commonly abused opioids such as heroin. While novel opioids may be structurally dissimilar, in general, these compounds target the μ-opioid receptor. Activation of this receptor causes analgesia, sedation, and euphoria which are the desired effects for users. Adverse effects include itching, nausea, constipation, and decreased respiration. Users develop tolerance to both the desired and undesired effects of opioid compounds, thus requiring larger and larger doses. The development of tolerance along with unknown identity of street drugs is a dangerous combination. The potency of these compounds varies greatly. Individuals who believe they are using heroin may use a dose consistent with previous doses that resulted in the desired effect, but the presence of another, more potent, opioid could make this dose deadly. Also of concern is the increased toxicity of opioids when combined with benzodiazepines. Taken alone prescription benzodiazepines rarely result in death, but the combination of benzodiazepines and opioids can have a synergistic effect on respiratory depression.

Overdose with novel opioids is assumed to be similar to that which is observed with any other μ-opioid receptor agonist and, like overdoses with heroin or prescription opiates such as oxycodone or oxymorphone, can be treated with naloxone. Naloxone acts as antagonist at the μ-opioid receptor. Since naloxone has a must stronger binding affinity for the receptor than opioid agonists, it can replace them at the receptor and block additional receptors, quickly and effectively reversing the central nervous system (CNS)

depression caused by opioids. Since naloxone is extremely safe, several states have made it available over-the-counter, and first responders are usually equipped with the drug as a mechanism of reducing opioid overdose-related deaths. This antidote is very much needed as novel opioids are being increasingly found in postmortem cases.

3.3 Benzodiazepines

Benzodiazepines are one of the most commonly prescribed psychotropic drug classes in the USA. There are 14 benzodiazepines available by prescription used to treat a variety of physical and psychological conditions. Compounds such as midazolam and triazolam have half-lives of 2–3 h and are often used to induce anesthesia or as sleep medication. Alprazolam, lorazepam, and clonazepam are common medications used to treat anxiety, panic disorders, and seizures with effects lasting for 6–10 h. Chlordiazepoxide which is used as a muscle relaxant and diazepam which is an anti-anxiolytic are two examples of long-acting benzodiazepines. These compounds typically have half-lives exceeding 24 h. The gamma-aminobutyric acid A receptor is the target receptor for benzodiazepines. This receptor has multiple binding sites which may explain the differences in degree and type of reactions observed from different benzodiazepines. All benzodiazepines have sedating effects making them a target for abuse. In general, the drugs are safe, but when combined with other CNS depressants, such as alcohol or opioids, the danger increases.

Novel benzodiazepines are often referred to as "research benzos" by drug users. Medications available in other countries but not legal prescription medications in the USA, and compounds which are not used in any country comprise this group. Phenazepam and etizolam were the first two benzodiazepines to appear in the illicit drug market in the USA. The DEA reports that between 2008 and 2013, there were 284 reports of phenazepam, and the drug was found in 31 states [9]. Etizolam appeared in 2012 with a total of 140 reports between its first appearance and June 2014 according to the DEA (14). Other compounds that have been reported include bromazepam, flubromazepam, flubromazelam delorazepam, diclazepam, and clonazelam.

Some signs and symptoms of benzodiazepine overdose include anxiety, agitation, dizziness, confusion, nystagmus, slurred speech, altered mental state, amnesia, hypotension, and impaired cognition. Respiratory depression may occur with benzodiazepine use, the degree of which will be significantly increased if used in combination with other central nervous system depressants. Flumazenil is a specific antidote for benzodiazepine overdose, but the risk of flumazenil may outweigh potential benefits, so it is ideally only used to treat acute overdose in the benzodiazepine naïve individual. Designer benzodiazepines are increasingly being detected in hospitalizations, impaired driving cases, and overdose deaths.

3.4 Other Classes

At the same time synthetic cannabinoids were making an appearance in the USA, synthetic cathinones and sympathomimetic amines – derivatives of cathinone and methamphetamine – were gaining in popularity. Commonly called "bath salts" or "plant food," the first generation of these products contained compounds such as MDPV, mephedrone, and methylone. Although the products were all classified under the same street name of "bath salts" and later "party pills" or "party powders," the types and activities of the compounds or combinations of compounds they contained varied greatly including stimulants, hallucinogens, entactogens, and dissociative anesthetics.

These substances are chemically similar to cathinone, a natural stimulant found in the khat plant, as well as amphetamine and methamphetamine, which are two stimulants that are routinely found as drugs of abuse. Small chemical changes to the structure of cathinone and/or amphetamine result in several new drugs that pharmacologically behave very similarly, producing the same spectrum of stimulant effects such as increased energy, tachycardia, and hypertension.

Like synthetic cannabinoids, trends in the popularity of these compounds can be traced to legislation controlling them. Following passage of the Synthetic Drug Abuse Prevention Act of 2012, the stimulant MDPV was replaced by alpha-PVP; reported "flakka" use soared between 2014 and 2015; however, after China was pressured by the USA to ban sale of number of chemicals, including alpha-PVP, the chemical has disappeared. Methylone was a popular entactogen among attendees at electronic music festivals but over time was replaced by ethylone followed by butylone and dibutylone.

2C-B and 5-MeO-DiPT are just two of the many psychedelic substances described by Alexander Shulgin in his drug tomes. Of the novel psychoactive substances that would be considered synthetic hallucinogens, most only have appeared in a handful of cases, with no one particular substance gaining significant popularity [10, 11].

Dissociative anesthetics such as methoxetamine and 3-methoxy-phencyclidine are structurally related to ketamine and phencyclidine and are reported to have similar effects. Though the mechanism of action is not completely understood, these compounds are antagonists at the NMDA receptor. Antagonism of this receptor results in hallucinations and dissociation.

4 Challenges of NPS

NPS present many challenges to clinical and forensic toxicologists primarily due to the rapid change in compounds being used. From an analytical perspective, the wide range of chemical structures and

classes comprising NPS means multiple tests must be run to determine what drug might be present. In addition, it is nearly impossible for a laboratory to maintain an up-to-date test due to lack of resources including analytical standards. Also, labs must weigh the high cost of developing a test for a new drug with the realization that the window of use might be narrow. By the time the test is ready and meets industry standards, users may have moved on to another substance. Labs must also be aware that new isomers of existing compounds may interfere with their existing test. For example, many of the fentanyl analogs have the same molecular weight and fragmentation patterns, so assays which employ short run times may not be able to resolve these isomers.

While the novel benzodiazepines and fentanyl-related substances might cross-react with routine urine drug screens, other types of NPS may not be detected in a patient arriving at the emergency department. Therefore, clinical toxicologists must treat patients suffering from toxic effects of drugs without knowing the specific compound. Additionally, most of these drugs have never been tested on animals, and there is limited information on their pharmacology. Therefore it is difficult to assess the role a designer drug has in a case because little to no information is available correlating presence or quantitation in biological fluids to effects on the human body.

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Chapter 14

Analysis of Synthetic Cannabinoid Metabolites by Liquid Chromatography-Tandem Mass Spectrometry

Gregory C. Janis

Abstract

The analysis of synthetic cannabinoid compounds in a urine sample is currently one of the more complex tasks facing toxicologists. The list of prevalent compounds in circulation at any given time is constantly in flux, changing at a rapid rate to avoid legal control and to a lesser extent to avoid detection. Even with knowledge of the chemical entities, their detection in urine is complicated by the fact that they are present exclusively as both phase I metabolites and phase II conjugates. With proper knowledge of the correct analytical targets, relatively simple procedures are capable of extracting and analyzing synthetic cannabinoids. Following enzymatic hydrolysis, compounds can be extracted through liquid partitioning procedures, and the extracts are analyzed via LC-MS/MS.

Key words Synthetic cannabinoids, Spice, K2, Urine drugs of abuse

1 Introduction

Few things have challenged analytical toxicology to the degree of synthetic cannabinoids. The inherent complexity of detecting xenobiotics is significantly more challenging when targeting any designer drugs, and targeting synthetic cannabinoids has raised the challenge several steps higher. The rapid proliferation of synthetic cannabinoids in combination with significant phase I metabolic transformations results in the field of analytical toxicology perpetually one step behind drug trends.

Synthetic cannabinoids first appeared as drugs of abuse in England in approximately 2004. At that time, the active compounds consisted primarily of JWH-018, a research tool originally developed by John Huffman of Clemson University [1] and later clandestinely produced as a street drug. JWH-018 is just one of a large family of structural derivatives possessing activity at one or more of the cannabinoid receptor subtypes. In addition to the chemical species related to JWH-018, multiple other families of

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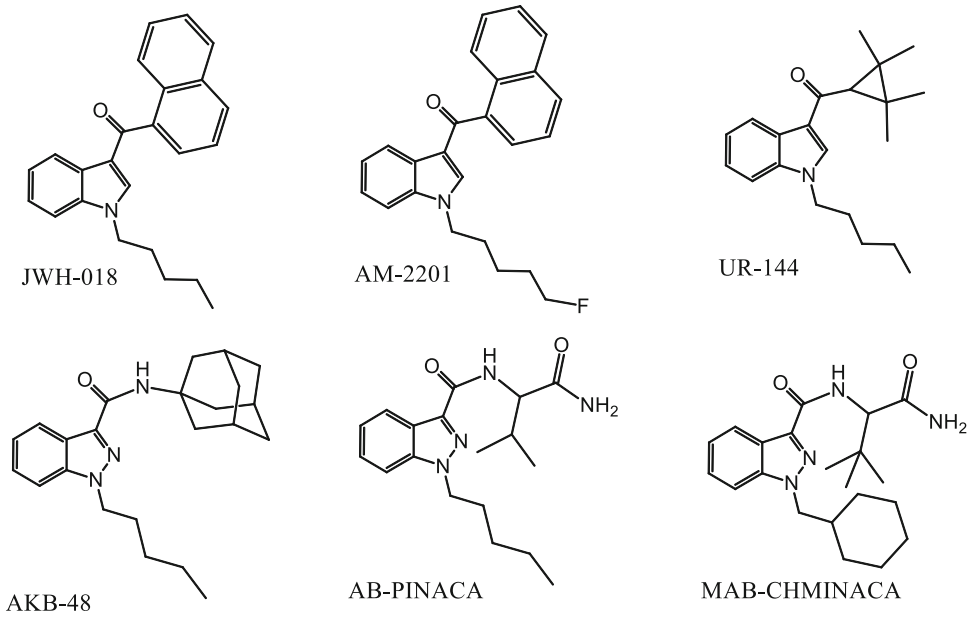

Fig. 1 Structures of representative synthetic cannabinoids

cannabinoid receptor agonists were developed as research tools or as potential drug candidates. However, since the original introduction of clandestinely produced JWH-018, clandestine chemistry has taken over developing new derivatives of synthetic cannabinoids for use as drugs of abuse with nearly wavelike consistency. The evolution of clandestine synthetic cannabinoids follows a relatively logical path of structural permutations. Minor permutations such as the addition of fluorine to known active structures are common. So are minor changes in aliphatic regions of active molecules including changing the length of carbon chains or cyclizing aliphatic chains. Some examples of the dominant synthetic cannabinoids over the decade are shown in Fig. 1. These drugs are typically applied to plant-based substrates with the intention of smoking the material similar to marijuana.

As neither animal nor human clinical trials were ever initiated on these compounds, there was no knowledge of the safety or metabolism of these drugs nor was there any knowledge of the metabolism or excretion of synthetic cannabinoids. Knowing the metabolism of these drugs is a prerequisite to urine sample analysis. Through forensic and in vitro testing, it was determined that JWH-018 and its most similar analogs were primarily metabolized via terminal oxidation of the aliphatic chain and subsequently further oxidized to a terminal carboxylic acid. Phase I metabolites are then subjected to phase II glucuronide conjugation [2, 3]. Un-metabolized parent synthetic cannabinoids do not generally

appear in urine samples; thus urine-based assays must target the proper metabolites for each targeted species.

The requirement for metabolic knowledge and corresponding reference standards is just one complication of synthetic cannabinoid analysis. Analysis is additionally challenging due to the constant evolution of these compounds. Historically, the typical lifespan of any specific synthetic cannabinoid as a drug of abuse is less than a year. In the period following a specific synthetic cannabinoid entering the drug scene, legal restrictions are applied and enforced. In response, clandestine manufacturing has historically switched to alternative analogs lacking legal restrictions. Testing laboratories must then identify the new chemical entity and, once again, predict and determine the most appropriate metabolites to analytically target.

The typical method of self-administering synthetic cannabinoids adds yet another layer of complexity for toxicological study. Many of the drug entities are structurally modified by pyrolysis [4, 5]. Thus, the chemical species a user is exposed to may not be the same species as found in the dosing product.

Despite the plethora of synthetic cannabinoids, some analytical strategies have proven to be applicable to the analysis of most compounds. Liquid chromatographic analysis is straightforward. Both C18 and C8 columns with typical gradients can produce adequate separations, although isobaric isomers exist within the family of drugs. One must also be aware that while the parent drugs are amines, predominant metabolites are often zwitterions (*see* Fig. 2). The existence of zwitterions does require the use of an acidic mobile phase to obtain sufficient ionization for positive ion mass spectrometry. Tandem mass spectrometry methods are set up using compound-specific fragmentation. Quantitation is performed against a concurrently analyzed calibration curve of the analytes of interest.

2 Materials

2.1 Solutions and Standards

1. 50:50 methanol/water (v/v): Add 100 mL of methanol and 100 mL of deionized water to a glass container, cap tightly, and mix. May be stored at room temperature for up to 6 months.
2. Analytical stock standards: 500 μg/mL of each drug. Prepare stock solutions of the analytical targets by accurately weighing 5 mg of each of the following reference standards into individual 10 mL volumetric flasks and diluting to volume with acetonitrile, AB-CHMINACA butanoic acid, AB-FUBINACA oxobutanoic acid, AB-PINACA pentanoic acid, ADBICA N-(4-hydroxypentyl), ADBICA pentanoic acid, ADB-PINACA pentanoic acid, AKB-48 N-pentanoic acid,

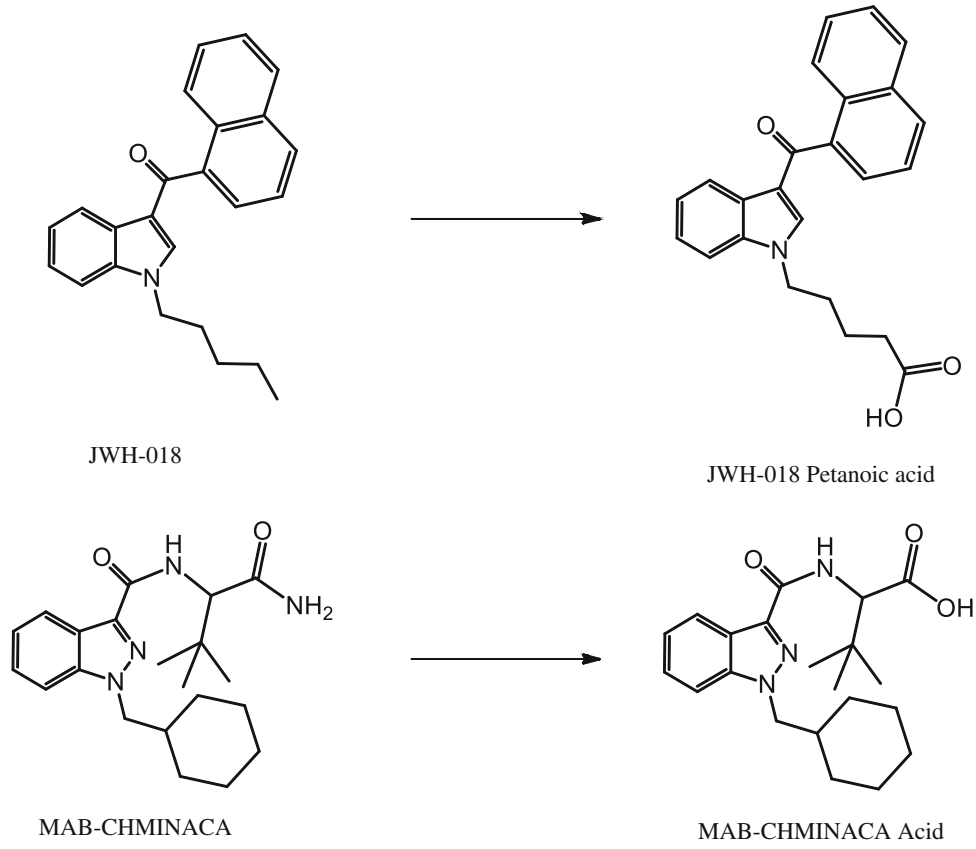

Fig. 2 Predominant metabolism of JHW-018 and MAB-CHIMACA

JWH-018 N-pentanoic acid, JWH-073 N-butanoic acid, MAB-CHMINACA butanoic acid, MAM2201 N-pentanoic acid, and UR-144 N-pentanoic acid.

3. 1.0 μg/mL combined working standard: Combine 20 μL of each of the 500 μg/mL stocks in a single 10 mL volumetric flask. Dilute to volume with 50:50 methanol/water (*see* **Note 1**).
4. 50 ng/mL combined working standard: Pipette 50 μL of the 1 μg/mL combined working standard into a 10 mL volumetric flask. Dilute to volume with 50:50 methanol/water.
5. Internal standard stock solutions: Weigh approximately 1 mg of each of the following isotopically labeled analogs into a 10 mL volumetric flask and dilute to volume with acetonitrile, JWH 073 N-butanoic acid D5, JWH 018 N-pentanoic acid D4, AM2201 N-(4-hydroxypentyl) D5, UR-144 N-pentanoic acid D5.
6. Internal standard spiking solution: 100 ng/mL of each internal standard. Aliquot 100 μL of each 100 μg/mL internal standard

stock in a 10 mL volumetric flask and dilute to volume with 50:50 methanol/water.

7. 50:50 hexane/ethyl acetate (v/v): Add 500 mL of hexane and 500 mL of ethyl acetate to a glass container, cap tightly, and mix. May be stored at room temperature for up to 6 months.
8. 0.1 N hydrochloric acid (HCl): Add approximately 40 mL of type I water to a 50 mL volumetric flask. Pipette 431 µL of concentrated HCl (35%, 11.6 M) to the flask. Bring to volume with reagent grade water. Transfer to a sealable glass bottle. Cap tightly and store at room temperature and is stable 1 year at ambient temperature.
9. 10 M potassium hydroxide: Weigh 560 g of solid potassium hydroxide pellets into a 1 L reagent bottle. Dissolve pellets in 500 mL of reagent grade water. Dilute with an additional 500 mL of reagent grade water. Cap tightly and store at room temperature and is stable 1 year at ambient temperature.
10. 50 mM potassium phosphate hydrolysis buffer, pH6.0: Add approximately 3600 mL of type 1 reagent grade water to a 4 L beaker. Add 13.6 mL of concentrated phosphoric acid (85%, 14.75 M) to the beaker with a graduated cylinder and mix. Adjust the pH to 5.0 with 10 M potassium hydroxide. Dilute to volume with type 1 water. Transfer to an amber reagent bottle and label with appropriate information. Cap tightly and store at room temperature and is stable 1 year at ambient temperature.
11. Mobile phase A, 10 mM ammonium acetate, 0.1% formic acid: Triple rinse a clean 1 L volumetric flask with type 1 laboratory grade water. Add approximately 500 mL of type I laboratory water to the volumetric flask. Weigh 0.77 g of high purity ammonium acetate (>99.99%) into a new reagent boat. Transfer the contents to the volumetric flask. Add 1.0 mL of high purity concentrated formic acid (>99%, 30 M) to the volumetric flask, dilute to volume with water, and mix. Stable when stored in a glass bottle at room temperature for up to 6 weeks.
12. Mobile phase B: Methanol, HPLC grade.
13. 50:50 methanol/ammonium acetate with formic acid: In a glass container, combine 250 mL of HPLC grade methanol and 250 mL of mobile phase A. Stable when stored at room temperature for up to 6 weeks.

2.2 Supplies and Equipment

1. β-Glucuronidase enzyme (*E. coli with approximately 140 U/mg of activity at 37 °C*).
2. Multi-tube vortex (VWR VX-2500 or similar).
3. Heat block for extraction tubes.
4. Nitrogen evaporator, e.g., TurboVap or similar.

5. Centrifuge (Beckman Coulter Allegra 25R, or similar).
6. LC-MS vials and caps.
7. Waters HSS T3, 50 × 2.1 mm, 1.8 μm column.
8. Thermo BetaBasic C18 guard column, 10 × 2.1 mm, 5 μm.
9. LC-MS/MS system (Waters classic UPLC and Sciex 5500 triple quadrupole).
10. 15 mL disposable glass round bottom tubes.
11. 10 mL disposable glass round bottom tubes.

3 Methods

3.1 Calibrator and Control Preparation

1. Pipette 0.500 mL of negative urine into labeled glass 15 mL extraction tubes for calibrators and controls.
2. Pipette 10 μL of the 0.05 μg/mL combined working standard into the tube labeled standard 1. Final concentration: 1.0 ng/mL.
3. Pipette 50 μL of the 0.05 μg/mL combined working standard into the tube labeled standard 2. Final concentration: 5.0 ng/mL.
4. Pipette 25 μL of the 1.0 μg/mL combined working standard into the tube labeled standard 3. Final concentration: 50.0 ng/mL.
5. Use standard solutions prepared from an independently prepared lineage of stock standards to prepare quality controls. Fortify 500 μL of negative urine with 30 μL of the 0.05 μg/mL combined standard for the low quality control. Spike 500 μL of negative urine with 20 μL of the 1.0 μg/mL combined standard for the high quality control.

3.2 Extraction

1. Pipette 0.5 mL of each sample into labeled 15 mL glass tubes.
2. Add 20 μL of the internal standard spiking solution into each calibrator, control, and sample tube.
3. Add 500 μL of 50 mM potassium phosphate buffer, pH 6 to each tube.
4. Add 20 μL of β-glucuronidase enzyme to each tube (*see* **Note 2**).
5. Vortex tubes at medium speed for 1 min.
6. Incubate samples at approximately 55 °C for 20 min.
7. Remove samples promptly and place at room temperature for approximately 10 min.
8. Add 1.0 mL of 0.1 N HCl to each tube and vortex mix samples for 1 min.

9. Add 5.0 mL of 50% hexane/50% ethyl acetate to each tube (*see* **Note 3**).
10. Cap and vortex mix tubes at low speed for 5 min while maintaining sample mixing.
11. Centrifuge tubes at 1300 g for 5 min.
12. Using disposable pipettes, transfer the upper, organic layer into properly labeled 15 mL disposable centrifuge tubes.
13. Dry the organic extracts under a stream of N2 while heating at approximately 45 °C for approximately 15 min, and remove promptly when dry.
14. Add 200 μL of 50:50 methanol and 10 mM ammonium acetate +0.1% formic acid to each tube.
15. Vortex tubes at high speed for 5 min.
16. Transfer extract into a properly labeled disposable glass autosampler vial with flat insert and cap tightly.

3.3 LC-MS/MS Parameters

1. Column temperature: 50 °C.
2. Flow rate: 0.7 mL/min.
3. Injection volume: 10 μL.
4. Autosampler temperature: 10 °C.
5. Gradient elution profile: Shown in Table 1.
6. Monitored transitions and analyte to internal standard pairs are listed in Table 2 (*see* **Note 4** and Fig. 3).

Table 1
UPLC gradient

Time (minute)	A%	B%	Flow (mL/min)
0.00	72	28	0.700
0.50	52	48	0.700
5.50	52	48	0.700
6.00	32	68	0.700
7.80	32	68	0.700
8.40	28	72	0.700
8.50	72	28	0.700
9.00	70	28	0.700

Table 2
Monitored tandem mass spectrometer transitions

Analyte/IS	Parent species	Precursor (*m/z*)	Quantitative fragment (*m/z*)	RT (min)	Collision energy	Paired internal standard
UR-144 *N*-pentanoic acid	UR-144/XLR-11	342.2	144.1	6.42	48	UR-144 N-pentanoic acid-D_5
JWH-073 N-butanoic acid	JWH-073	358.0	155.0	4.06	30	JWH 073 N-butanoic acid-D_5
AB-CHMINACA butanoic acid	AB-CHMINACA	358.3	241.1	7.05	25	UR-144 N-pentanoic acid-D_5
ADBICA N-(4-hydroxypentyl)	ADBICA	360.1	156.1	4.06	30	AM2201 N-(4-hydroxypentyl)-D_5
AB-PINACA pentanoic acid	AB-PINACA and F-AB-PINACA	361.1	217.0	1.37	43	RCS-4 N-pentanoic acid-D_5
JWH-018 N-pentanoic acid	JWH-018 and AM2201	372.0	155.0	5.39	27	JWH 018 N-pentanoic acid-D_4
ADBICA pentanoic acid	ADBICA	374.2	244.1	2.01	30	RCS-4 N-pentanoic acid-D_5
ADB-PINACA pentanoic acid	ADB-PINACA	375.3	245.2	2.01	32	RCS-4 N-pentanoic acid-D_5
MAM-2201 N-pentanoic acid	MAM-2201	386.2	141.1	6.36	54	JWH 398 N-pentanoic acid-D_5
AKB-48 N-pentanoic acid	AKB-48 and 5F-AKB-48	396.3	107.1	7.11	62	UR-144 N-pentanoic acid-D_5
AB-FUBINACA oxobutanoic acid	AB-FUBINACA	399.3	253.3	1.87	28	RCS-4 N-pentanoic acid-D_5
MAB-CHMINACA butanoic acid	MAB-CHMINACA	372.6	145.0	7.70	52	UR-144 N-pentanoic acid-D_5
JWH 073 N-butanoic acid-D_5	–	363.2	155.1	3.96	33	
RCS-4 N-pentanoic acid-D_5	–	357.2	135.1	2.85	45	
UR-144 N-pentanoic acid-D_5	–	347.3	125.0	6.41	30	
AM2201 N-(4-hydroxypentyl)-D_5	–	381.3	155.1	4.57	45	
JWH 018 N-pentanoic acid-D_4	–	376.3	155.1	5.35	30	

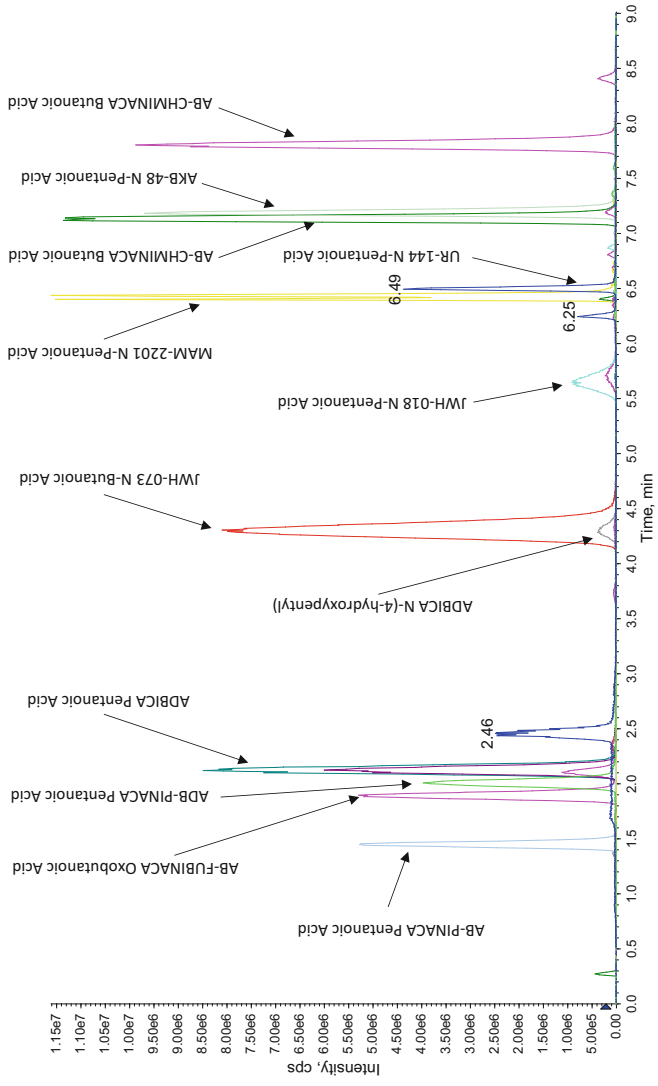

Fig. 3 Representative chromatogram

4 Notes

1. When preparing calibrators and quality control samples, one must keep in mind the poor water solubility of the free drugs and metabolites. Insolubility can result in analyte losses during storage. Calibrators and quality controls should be kept frozen when not in use. This is less of a concern for actual samples as phase II conjugation significantly improves water solubility.
2. In urine, phase II conjugates must be hydrolyzed releasing free drug phase II metabolites. Attempts to analyze intact conjugates in urine are not practical as few reference standards are available for the conjugated drugs. Both chemical and enzymatic hydrolysis methods have been employed. However, some more recent compounds contain ester and amide moieties; these compounds may degrade at elevated pH. Thus, enzymatic hydrolysis is typically preferred. Beta glucuronidase from abalone and *E. coli* can both be used successfully; however, recombinant *E. coli* has been shown to be particularly efficient. With the addition of *E. coli* derived beta glucuronidase, phase II glucuronide conjugates can be efficiently hydrolyzed in as little as 30 min with mild incubation.
3. Like tetrahydrocannabinol (THC) and its primary urine metabolite carboxy-THC, unconjugated synthetic cannabinoids are significantly nonpolar. Thus, liquid-liquid extraction is capable of easily extracting the drugs and unconjugated metabolites. Mixtures of hexane and ethyl acetate have proven to extract metabolites of synthetic cannabinoid from urine with a high degree of efficiency while avoiding significant analytical interference. The organic extracts only need to be dried and reconstituted prior to LC-MS/MS analysis. Solid-phase techniques, again exploiting the nonpolar nature of the drugs, have also been successfully employed for extraction of synthetic cannabinoid metabolites.
4. Quantitation of donor samples is achieved by comparing the analyte to internal standard response of detected peaks to the generated calibration curve for the corresponding analyte. Utilize a $1/x$ weighted linear regression for all analytes.

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Chapter 15

Quantification of Designer Opioids by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

Opioids including heroin and commonly prescribed drugs such as oxycodone and fentanyl are among the most commonly abused drugs. In recent years, the abuse of opioids has spread beyond these commonly encountered analytes and now includes novel psychoactive drugs such as AH-7921 and U47700 and a variety of fentanyl-related compounds such as acetyl fentanyl and furanyl fentanyl. The assay described is for the quantitative determination of 19 designer opioids in serum, plasma, and whole blood. Also included is a discussion on the challenges of keeping an analytical method current as new analytes appear on the illicit drug market.

Key words Designer opioids, Novel opioids, Research opioids, LC-MS/MS

1 Introduction

Chapter 13 provides a brief history of designer opioids. Analysis of biological matrices for designer opioids by liquid chromatography–tandem mass spectrometry (LC-MS/MS) generally does not present an analytical challenge for laboratories with experience in analyzing these types of specimens for classical opioids. Rather, the challenge is the availability of certified reference materials and deuterated internal standards and the variety of chemical structures that comprise designer opioids. It is also important to be forward thinking during method development so that the final method can be quickly updated to include new analytes. While there are a few published gas chromatography-mass spectrometry (GCMS) methods for designer opioids including acetyl fentanyl and butyryl fentanyl [1–4], the preferred method for quantification of designer opioids appears to be LC-MS/MS [5–14]. In general, analytes of interest are extracted from biological specimens using either liquid-liquid or solid-phase extraction before being introduced in a LC-MS/MS system.

Loralie J. Langman and Christine L.H. Snozek (eds.), *LC-MS in Drug Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1872, https://doi.org/10.1007/978-1-4939-8823-5_15,

The presented method is a modification of a validated method for MT-45, butyryl fentanyl, AH-7921, and acetyl fentanyl in serum, plasma, and whole blood. The original method had a run time of only 5.00 min (min) during which all four analytes were completely resolved. However, after approximately 5 months of routine use in a large reference laboratory, a large interfering peak appeared in the window for AH-7921. After identifying the interfering substance as U47700, minor method modifications allowed for the separation of AH-7921 and U47700. At the same time, new fentanyl analogs were added to the analytical method. An extended run time of 6.00 min allows for the resolution of the majority of analytes; two pairs of isomers are resolved. Calibrators are prepared with a mixed analyte spiking solution prepared in serum, while bulk controls are prepared in serum, frozen and diluted with whole blood at the time of analysis. Solid-phase extraction is used to clean up and concentrate the samples. Separation is achieved using a Zorbax Rx-SIL normal phase analytical column with gradient elution by Waters Acquity ultra performance liquid chromatography system. A Waters TQD tandem mass spectrometer with a positive-ion electrospray source is used to identify and quantify analytes of interest. Table 1 lists the analytical ranges for all compounds included in the panel.

2 Materials

2.1 Prepared Reagents

All reagents are reagent grade or better.

1. 5% ammonium hydroxide in acetonitrile (ACN): In the fume hood, add 5.0 mL of ammonium hydroxide and 95 mL of ACN to a 100 mL bottle; mix thoroughly. Prepare fresh daily.
2. 0.1% formic acid in ACN: Transfer 999 mL of ACN to a 1.0 L glass bottle. Add 1.0 mL of formic acid. Cap and mix by inversion.
3. 0.1% formic acid in deionized (DI) water: Transfer 999 mL of DI water to a 1.0 L glass bottle. Add 1.0 mL of formic acid. Cap and mix by inversion.
4. 0.1 N hydrochloric acid (HCl): Transfer 8.4 mL of HCl to a 1.0 L volumetric flask containing approximately 400 mL of DI water. Dilute to volume with DI water; mix thoroughly.
5. 0.1 M sodium phosphate buffer, pH 6.0: Add 3.4 g of sodium phosphate dibasic and 21.1 g of sodium phosphate monobasic to approximately 200 mL of DI water in a 2.0 L volumetric flask. Swirl to dissolve and dilute to volume with DI water. Adjust pH to 6.0 with 0.5 M sodium phosphate dibasic. Mix thoroughly.

Table 1
Designer opioid analytes

Analyte	Analytical range (ng/mL)	Stock concentration	Group
4-ANPP	0.1–10	Powder	A
4-Methoxybutyryl fentanyl	0.1–10	Powder	A
4-Methylphenethyl acetyl fentanyl	0.1–10	Powder	A
Acryl fentanyl	0.1–10	Powder	A
Alpha-methyl fentanyl	0.1–10	Powder	A
Butyryl fentanyl[a]	0.1–10	Powder	A
Carfentanil	0.1–10	1000 ng/mcL	A
Furanyl fentanyl	0.1–10	Powder	A
MT-45	0.1–10	1000 ng/mcL	A
Ortho-fluorofentanyl	0.1–10	Powder	A
Para-fluorobutyryl fentanyl[a]	0.1–10	Powder	A
Para-fluorofentanyl	0.1–10	Powder	A
Valeryl fentanyl	0.1–10	Powder	A
AH-7921	0.2–20	1000 ng/mcL	B
U-47700	0.2–20	Powder	B
U-50488	0.2–20	Powder	B
Beta-hydroxythiofentanyl	0.5–50	Powder	C
D_5-Beta-hydroxythiofentanyl	IS	Powder	
D_5-Para-fluorofentanyl	IS	Powder	
D_3-Alpha-methyl fentanyl	IS	Powder	
D_5-Methylphenethyl acetyl fentanyl	IS	Powder	
13C6-Acetyl fentanyl	IS	50 ng/mcL	
D_6-U-47700	IS	Powder	
D_3-AH-7921	IS	100 ng/mcL	

[a]Some analytes are not resolved by this method and are reported as indicated: butyryl/isobutyryl fentanyl and para-fluorobutyryl fentanyl/para-fluoroisobutyryl fentanyl (FIBF). *IS* internal standard

6. 0.5 M sodium phosphate dibasic: Transfer 35.5 g of sodium phosphate dibasic to a 500 mL volumetric flask. Dissolve and dilute to volume with DI water. Mix thoroughly.
7. Mobile phase A, ammonium formate, pH 4.0: Transfer 900 mL of DI water to a 1.0 L beaker. Add 2 mL of formic acid, and adjust to pH 4.0 with approximately 2 mL of

ammonium hydroxide while mixing. Dilute to volume with DI water, and mix thoroughly.

8. Mobile phase B: HPLC-grade ACN.

2.2 Calibrators

See Table 1 for information on certified reference materials (CRM) used to prepare calibrators, internal standard (IS), and controls.

1. 100 ng/μL individual stock standards: Transfer 1.0 mg of 4-ANPP and U-47700, 1.2 mg of U-50488, and 1.1 mg of the remaining powdered CRM drugs, each to their own 10 mL volumetric flasks (*see* **Note 1**). Dilute to volume with methanol (MeOH). Store in amber glass vials with Teflon-lined caps. Unless otherwise noted, all stock solutions are stable for 1 year when stored in appropriate container at <−10 °C.
2. 100 ng/μL MT-45 and carfentanil substock standards: Combine 100 μL of each 1000 ng/μL stock of MT-45 or carfentanil with 900 μL of MeOH. Discard immediately after use.
3. Mixed bulk standard 1: 100 ng/mL Group A drugs, 200 ng/mL Group B drugs, and 500 ng/mL Group C drug. Transfer 50 μL of each 100 ng/μL Group A drug stock, 10 μL of the 1000 ng/mL AH-7921 stock, 100 μL of each remaining 100 ng/μL Group B drug stock, and 250 μL of the 100 ng/μL beta-hydroxythiofentanyl stock to a plastic 50 mL volumetric flask, and dilute to volume with drug-free human serum. Transfer 0.3 mL aliquots to labeled 2.0 mL plastic snap-cap conical tubes for storage in freezer (*see* **Note 2**).
4. Intermediate 10× calibrator 1: Thaw one tube of mixed bulk standard 1, and vortex briefly to mix. The undiluted mixed bulk standard is used as 10× calibrator 1.
5. Intermediate 10× calibrator 2: Transfer 200 μL of intermediate 10× calibrator 1 and 200 μL of drug-free serum to a 12 × 75 mm test tube. Mix well. Discard immediately after use. Concentrations of the 10× calibrators are shown in Table 2.
6. Intermediate 10× calibrator 3: Transfer 200 μL of intermediate 10× calibrator 2 and 300 μL of drug-free serum to a 12 × 75 mm test tube. Mix well. Discard immediately after use.
7. Intermediate 10× calibrator 4: Transfer 200 μL of intermediate 10× calibrator 3 and 600 μL of drug-free serum to a 12 × 75 mm test tube. Mix well. Discard immediately after use.
8. Intermediate 10× calibrator 5: Transfer 200 μL of intermediate 10× calibrator 4 and 300 μL of drug-free serum to a 12 × 75 mm test tube. Mix well. Discard immediately after use.
9. Intermediate 10× calibrator 6: Transfer 200 μL of intermediate 10× calibrator 5 and 200 μL of drug-free serum to a 12 × 75 mm test tube. Mix well. Discard immediately after use.

Table 2
Intermediate 10× and working calibrators

Calibrator	Prepared concentration (ng/mL)		
	Group A[a]	Group B	Group C
10× cal 1	100	200	500
10× cal 2	50	100	250
10× cal 3	20	40	100
10× cal 4	5.0	10	25
10× cal 5	2.0	4.0	10
10× cal 6	1.0	2.0	5.0
Working cal 1	10	20	50
Working cal 2	5	10	25
Working cal 3	2	4	10
Working cal 4	0.5	1.0	2.5
Working cal 5	0.2	0.4	1.0
Working cal 6	0.1	0.2	0.5

[a]Drugs are categorized as Groups A, B, and C as noted in Table 1

10. Working calibrators: Transfer 50 µL of each intermediate 10× calibrator to a labeled 13 × 100 mm test tube. Add 450 µL of drug-free human whole blood preserved with potassium oxalate/sodium fluoride (KOx/NaF) to each tube, and mix well. Table 2 lists the final concentration of the working calibrators.

2.3 Controls and Internal Standards (IS)

1. 100 ng/µL individual IS stock standards: Transfer 1.0 mg of d_6-U-47700 and 1.1 mg of the remaining powdered IS CRMs (d_5-beta-hydroxythiofentanyl, d_5-para-fluorofentanyl, d_3-alpha-methyl fentanyl, d_5-methylphenethyl acetyl fentanyl), each to its own 10 mL volumetric flask. Dilute to volume with MeOH and mix well. Store in amber glass vials with Teflon-lined caps.
2. Working IS: Add 40 µL each of d_3-alpha-methyl fentanyl, d_5-para-fluorofentanyl, and d_5-4-methylphenethyl acetyl fentanyl, 80 µL each of 13C6-acetyl fentanyl and d_6-U-47700, and 200 µL each of d_3-AH-7921 and d_5-beta-hydroxythiofentanyl to a 200 mL volumetric flask. Dilute to volume with MeOH. Store in amber glass bottle with Teflon-lined cap. Preparation instructions and final concentrations are shown in Table 3.
3. Carryover check control stock solution: 5.0 ng/µL furanyl fentanyl and 4-ANPP and 10 ng/µL U-47700. Transfer 0.5 mL of 100 ng/µL furanyl fentanyl stock solution, 0.5 mL

Table 3
Preparation of working internal standard solution

Analyte	Stock concentration (ng/μL)	Amount to add (μL)	Final concentration (ng/μL)
13C6-Acetyl fentanyl	50	80	0.02
D_3-AH-7921	100	200	0.1
D_3-Alpha-methyl fentanyl	100	40	0.02
D_5-Para-fluorofentanyl	100	40	0.02
D_5-Beta-hydroxythiofentanyl	100	200	0.1
D5-4-Methylphenethyl acetyl fentanyl	100	40	0.02
D_6-U-47700	100	80	0.04

of 100 ng/μL 4-ANPP stock solution, and 1.0 mL of 100 ng/μL U-47700 stock solution to a 10 mL volumetric flask. Dilute to volume with MeOH. Store in an amber bottle with Teflon-lined cap.

4. Working carryover check control: 150 ng/mL furanyl fentanyl and 4-ANPP, 300 ng/mL U-47700. Combine 15 μL of carryover check control stock solution and 0.5 mL of drug-free blood in a 13 × 100 mL test tube. Mix well. Sample is now ready for extraction.
5. 1 mg/mL individual stock quality control (QC) solutions: Transfer 10 mg of 4-ANPP and U-47700, 12 mg of U-50488, and 11 mg of the remaining powdered CRM drugs, each to their own 10 mL volumetric flasks. Dilute to volume with MeOH. Store in amber glass vial with Teflon-lined cap.
6. Mixed substock QC solution: 10 ng/μL Group A drugs, 20 ng/μL Group B drugs, and 50 ng/μL Group C drug. Transfer 100 μL of each individual Group A QC stock solution, 200 μL of each individual Group B QC stock solution, and 500 μL of beta-hydroxythiofentanyl QC stock solution to a 10 mL volumetric flask. Dilute to volume with MeOH. Store in amber glass vial with Teflon-lined cap.
7. Bulk high control: Transfer 175 μL of mixed substock QC solution to a plastic 25 mL volumetric flask, and dilute to volume with drug-free serum. Transfer 0.2 mL aliquots to labeled 2.0 mL plastic snap-cap conical tubes for storage in freezer (stable for 6 months). Table 4 lists the concentration of each analyte in the bulk low, bulk high, low working, and high working controls.

Table 4
Concentration of bulk 10× and working low and high controls

	Concentration (ng/mL)			
Analyte	Bulk 10× high	Bulk 10× low	Working low	Working high
4-ANPP	100	3.5	0.35	7.0
4-Methoxybutyryl fentanyl	100	3.5	0.35	7.0
4-Methylphenethyl acetyl fentanyl	100	3.5	0.35	7.0
Acryl fentanyl	100	3.5	0.35	7.0
Alpha-methyl fentanyl	100	3.5	0.35	7.0
Butyryl fentanyl/isobutyryl fentanyl	100	3.5	0.35	7.0
Carfentanil	100	3.5	0.35	7.0
Furanyl fentanyl	100	3.5	0.35	7.0
MT-45	100	3.5	0.35	7.0
Ortho-fluorofentanyl	100	3.5	0.35	7.0
Para-fluorobutyryl fentanyl/FIBF	100	3.5	0.35	7.0
Para-fluorofentanyl	100	3.5	0.35	7.0
Valeryl fentanyl	100	3.5	0.35	7.0
AH-7921	200	7.0	0.7	14
U-47700	200	7.0	0.7	14
U-50488	200	7.0	0.7	14
Beta-hydroxythiofentanyl	500	17.5	1.75	35

8. Bulk low control: Transfer 1250 μL of bulk high control to a plastic 25 mL volumetric flask, and dilute to volume with drug-free serum. Transfer 0.2 mL aliquots to labeled 2.0 mL plastic snap-cap conical tubes for storage in freezer (stable for 6 months).
9. Low and high working controls (prepared at time of analysis): Thaw one bulk high control and one bulk low control. Transfer 50 μL of each bulk control to its own individually labeled 13 × 100 mm tube. Add 450 μL drug-free blood and mix well.

2.4 Supplies and Instrumentation

1. Agilent Plexa PCX 3.0 mL/60 mg solid-phase extraction (SPE) columns.
2. Waters TQD tandem mass spectrometer with Waters Acquity ultra performance liquid chromatography system.
3. Zorbax Rx-SIL 3 × 100 mm, 1.8 μm analytical column with Optimize EXP filter, 0.2 μm.

4. Vacuum manifold for SPE.
5. 12 × 75 mm and 13 × 100 mm test tubes.
6. Amber bottles with Teflon-lined caps.
7. Amber glass vials with Teflon-lined caps.
8. 2.0 mL plastic snap-cap conical tubes.
9. Volumetric glassware.
10. Pipettes and tips.
11. Vortexer.
12. Centrifuge with adaptors for 13 × 100 mm tubes.
13. Nitrogen evaporator.

3 Methods

3.1 Sample Preparation

1. Prepare working calibrators and controls as described above (*see* **Note 3**).
2. Transfer 0.5 mL of blank blood (for matrix blank), DI water (reagent blank), and each patient specimen to their own appropriately labeled 13 × 100 mm test tubes (*see* **Note 4** for samples with expected concentrations above the analytical range).
3. Add 25 μL working IS solution to each tube; vortex briefly to mix.
4. Add 1.0 mL of HPLC-grade ACN to each tube. Vortex for approximately 10 s to mix.
5. Centrifuge all test tubes at 2100×*g* for approximately 10 min.
6. Transfer supernatant to new, appropriately labeled 13 × 100 mm test tubes.
7. Add 1.0 mL of 0.1 M sodium phosphate buffer, pH 6.0, to each test tube; vortex briefly to mix.

3.2 Solid-Phase Extraction

1. Place one SPE column for each sample to be extracted in a vacuum manifold rack.
2. Condition columns with 2.0 mL of HPLC-grade ACN; aspirate slowly through column.
3. Equilibrate columns with 2.0 mL of DI water; aspirate through column. Do not allow columns to dry before application of samples.
4. Transfer prepared samples to columns; aspirate slowly.
5. Rinse columns with 2.0 mL of 0.1 N HCl. Follow by rinsing with 2.0 mL of ACN; aspirate slowly.
6. Place new, labeled glass tubes beneath each SPE column. Add 2.0 mL of 5% ammonium hydroxide in ACN to elute by gravitational flow.

7. Evaporate to dryness at 55 ± 5 °C under nitrogen flow.
8. Reconstitute in 500 µL of 0.1% formic acid in ACN; vortex thoroughly for approximately 30 s.
9. Transfer to appropriately labeled 0.5 mL plastic autosampler vials, and cap with Teflon-lined snap caps.

3.3 LC-MS/MS Analysis

1. Place samples in autosampler in the following order:
   (a) Reagent blank.
   (b) Working carryover check control.
   (c) Matrix blank.
   (d) Calibrators from highest to lowest (*see* **Note 5**).
   (e) High control.
   (f) Patient samples (bracketed by controls).
   (g) Low control.
2. Inject 20 µL in full loop mode.
3. Column temperature: 40 °C.
4. Flow rate: constant 0.6 mL/min.
5. Mobile phase ratio: 10% A, 90% B. No gradient.
6. MS parameters (should be optimized for instrument used):
   (h) Polarity: positive mode.
   (i) Capillary voltage: 0.50 kV.
   (j) Cone voltage: 35.00 V.
   (k) Extractor voltage: 3.00 V.
   (l) RF voltage: 0.10 V.
   (m) Source temperature: 120 °C.
   (n) Desolvation temperature: 450 °C.
   (o) Cone gas flow: 35 L/h.
   (p) Desolvation gas flow: 1000 L/h.
   (q) Collision gas flow: 0.15 mL/min.
   (r) MSMS mode entrance: −5.00.
   (s) MSMS mode collision energy: 16.00.
   (t) MSMS mode exit: 1.00.
   (u) Gain: 1.00.
7. Data acquisition parameters are outlined in Table 5 (*see* **Note 6**).
8. Figure 1 portrays a calibrator containing all analytes and IS, with an expanded view in Fig. 2. Two transitions are monitored for each analyte (Table 6).

Table 5
Data acquisition parameters

Time (min)	Channel	Reaction	Dwell (secs)	Cone volt.	Col. energy
1.400–2.400	1	359.20 > 111.00	0.007	35	34
	2	359.20 > 192.10	0.007	35	22
	3	364.20 > 111.00	0.007	35	34
	4	364.20 > 192.10	0.007	35	22
1.500–2.500	1	395.30 > 113.00	0.007	40	34
	2	395.30 > 335.20	0.007	40	18
1.700–2.700	1	365.20 > 105.00	0.006	45	36
	2	365.20 > 188.20	0.006	45	24
	1	369.30 > 105.10	0.006	50	38
	2	369.30 > 188.10	0.006	50	24
	1	375.10 > 105.00	0.006	45	40
	2	375.10 > 188.10	0.006	45	24
1.800–2.800	1	355.30 > 105.10	0.006	48	38
	2	355.30 > 188.10	0.006	48	24
	3	360.30 > 105.10	0.006	48	38
	4	360.30 > 188.10	0.006	48	24
	1	281.20 > 105.00	0.006	35	30
	2	281.20 > 188.20	0.006	35	16
1.900–2.900	1	335.20 > 105.00	0.006	45	32
	2	335.20 > 188.20	0.006	45	22
	1	351.10 > 105.00	0.006	45	36
	2	351.10 > 188.00	0.006	45	22
2.000–3.000	1	381.20 > 105.00	0.006	45	38
	2	381.20 > 188.20	0.006	45	24
2.100–3.100	1	349.10 > 169.00	0.006	45	18
	2	349.10 > 181.00	0.006	45	22
2.200–3.200	1	351.20 > 91.10	0.006	45	38
	2	351.20 > 202.10	0.006	45	22
	3	354.20 > 91.10	0.006	45	38
	4	354.20 > 202.10	0.006	45	22

(continued)

Table 5 (continued)

Time (min)	Channel	Reaction	Dwell (secs)	Cone volt.	Col. energy
2.500–3.500	1	337.20 > 119.00	0.006	45	34
	2	337.20 > 202.20	0.006	45	22
	3	342.20 > 119.00	0.006	45	34
	4	342.20 > 202.20	0.006	45	22
	1	323.10 > 105.00	0.006	45	36
	2	323.10 > 188.00	0.006	45	22
	3	329.10 > 105.00	0.006	45	36
	4	329.10 > 188.00	0.006	45	22
3.200–5.400	1	329.20 > 204.10	0.03	45	22
	2	329.20 > 284.10	0.03	45	18
	3	335.20 > 173.00	0.03	45	28
	4	335.20 > 284.10	0.03	45	18
3.600–5.400	1	329.10 > 173.00	0.03	45	26
	2	329.10 > 284.00	0.03	45	16
	3	334.10 > 178.00	0.03	45	26
	4	334.10 > 289.00	0.03	45	16
4.100–5.400	1	369.20 > 112.00	0.03	45	30
	2	369.20 > 298.10	0.03	45	20

4 Notes

1. Many of the powdered CRM drugs are sold as hydrochloride salts. The calculations are adjusted to ensure that the stock standard concentrations reflect the molecular weight of the free base rather than the hydrochloride salt. Similar adjustment is done for the QC and internal standard measurements.
2. The described calibrator and control preparation scheme allows for preparation of bulk material that can be frozen and used for at least 6 months. For bulk preparation, serum demonstrated better reproducibility than preparing the bulk calibrators and controls in whole blood. Intermediate 10× and working calibrators are all prepared at the time of analysis. Controls can be prepared using different sources or lot numbers than the standards; however, with novel drugs, multiple sources or lots might not be available. If different sources or lot numbers are

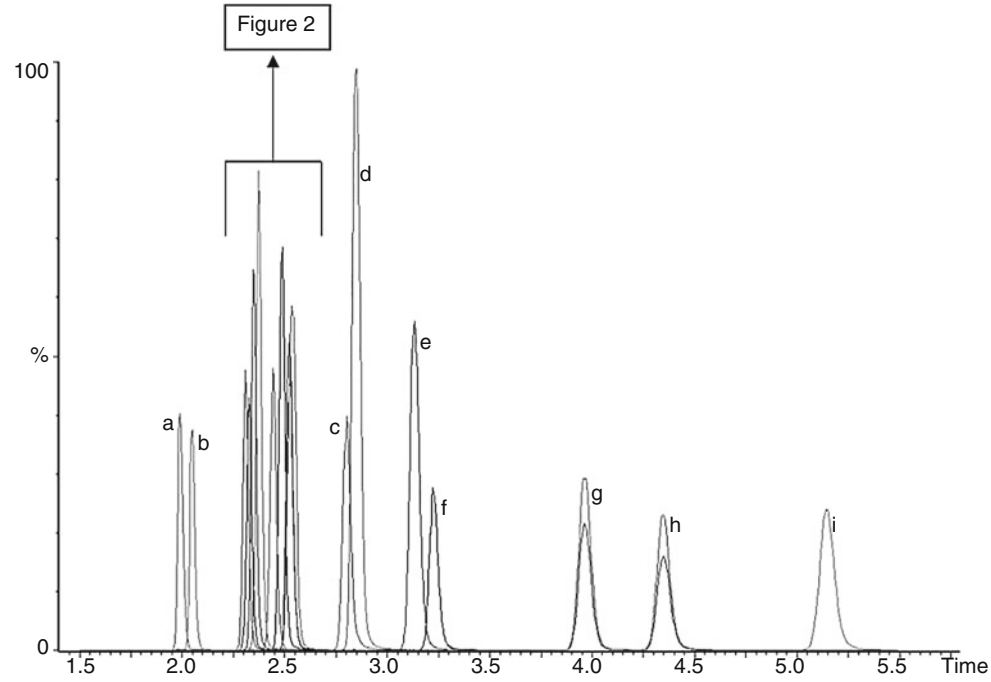

Fig. 1 Multiple reaction monitoring (MRM) chromatogram of extracted whole blood fortified with 10–50 ng/mL of each compound (**a**) beta-hydroxythiofentanyl, (**b**) carfentanil, (**c**) MT-45, (**d**) alpha-methyl fentanyl, (**e**) 4-methylphenethyl acetyl fentanyl, (**f**) acetyl fentanyl, (**g**) U-47700, (**h**) AH-7921, (**i**) U-50488

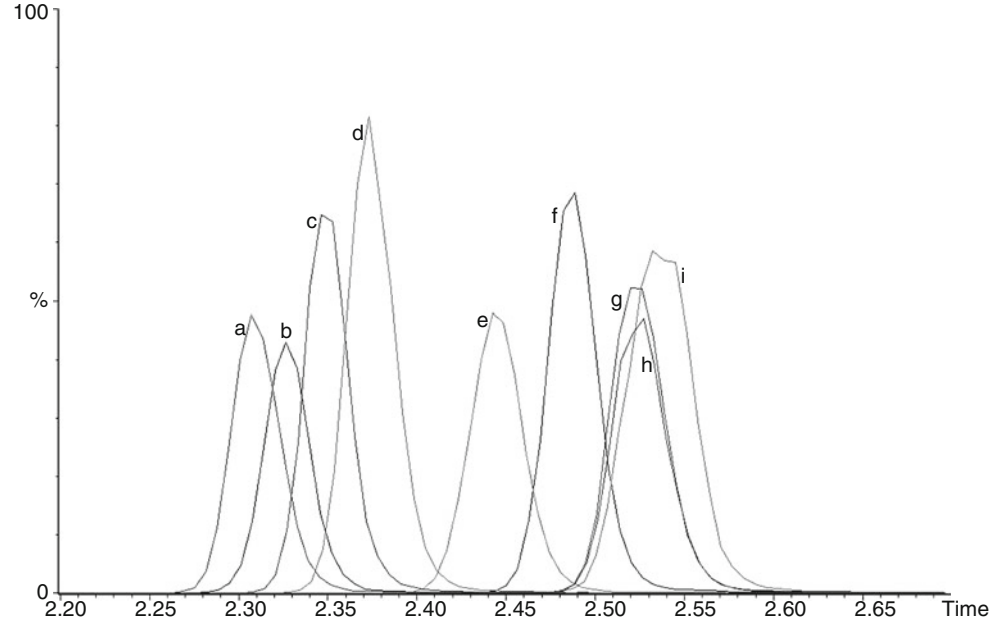

Fig. 2 Expanded view of Fig. 2 across 2.20–2.70 min (**a**) valeryl fentanyl, (**b**)para-fluorobutyryl fentanyl/FIBF, (**c**) 2-furanyl fentanyl, (**d**) ortho-fluorofentanyl, (**e**) 4-ANPP, (**f**) butyryl fentanyl/isobutyryl fentanyl, (**g**) acryl fentanyl, (**h**) 4-methoxybutyryl fentanyl, (**i**) para-fluorofentanyl

Table 6
Analyte retention times and monitored MRM transitions[a]

Analyte	Retention time (min)	Quantifier transition	Qualifier transition
Beta-hydroxythiofentanyl	1.92	359.2 > 192.1	359.2 > 111
D_5-Beta-hydroxythiofentanyl	1.93	364.2 > 192.1	364.2 > 111.0
Carfentanil	1.94	395.3 > 335.2	395.2 > 113.0
Valeryl fentanyl	2.17	365.2 > 188.2	365.2 > 105.0
Para-fluorobutyryl fentanyl	2.19	369.3 > 188.1	369.3 > 105.1
Furanyl fentanyl	2.22	375.1 > 188.1	375.1 > 105.0
Ortho-fluorofentanyl	2.23	355.3 > 105.1	355.3 > 188.1
4-ANPP	2.28	281.2 > 105.0	281.2 > 188.2
Butyryl fentanyl/isobutyryl fentanyl	2.33	351.1 > 105.0	351.1 > 188.0
4-Methoxybutyryl fentanyl	2.36	381.2 > 105.0	381.2 > 188.2
Para-fluorofentanyl	2.37	355.3 > 105.1	355.3 > 188.1
Acryl fentanyl	2.37	335.2 > 105.0	335.2 > 188.2
D_5-Para-fluorofentanyl	2.39	360.3 > 105.1	360.3 > 188.1
MT-45	2.55	349.1 > 181.0	349.1 > 169.0
Alpha-methyl fentanyl	2.62	351.2 > 91.1	351.2 > 202.1
D_3-Alpha-methyl fentanyl	2.63	354.2 > 91.1	354.2 > 202.1
4-Methylphenethyl acetyl fentanyl	2.88	337.2 > 119.0	337.2 > 202.2
D_5-4-Methylphenethyl acetyl fentanyl	2.90	342.2 > 119.0	342.2 > 202.2
13C6-Acetyl fentanyl	2.96	329.1 > 188.0	329.1 > 105.0
U-47700	3.61	329.2 > 284.1	329.2 > 204.1
D_6-U-47700	3.95	335.2 > 284.1	335.2 > 173.0
AH-7921	3.95	329.1 > 284.0	329.1 > 173.0
D_3-AH-7921	3.96	334.1 > 289.0	334.1 > 178.0
U-50488	4.63	369.2 > 298.1	369.2 > 112.0

[a]Analytes listed below IS used for quantification

not available controls should be prepared by a different individual or at a different time from the same materials as calibration standards.

3. Enough low and high controls should be prepared to bracket patient samples as required by laboratory standard operating procedures. A general rule of thumb is that fortified controls

should comprise at least 5% of the samples being analyzed and they should be interspersed throughout the run with no more than ten patient specimens between each control sample.

4. Samples that are expected to be above the analytical range for one or more analytes can be diluted with blank whole blood. A dilution control is not necessary because the working controls are prepared by diluting the 10× control. If more than 10× dilution of a patient specimen is required, an appropriate dilution control should be prepared by applying the sample dilution factor to the 10× control.
5. If no carryover check control is used, inject the matrix blank after the highest standard to check for carryover.
6. Acquisition parameters should be optimized for the instrumentation employed. The method, as written, has the following limitations:
   (a) Butyryl fentanyl and isobutyryl fentanyl are co-eluting isobaric compounds and cannot be distinguished based on this methodology. Results are reported as the pair.
   (b) Para-fluorobutyryl fentanyl and para-fluoroisobutyryl fentanyl (FIBF) are co-eluting isobaric compounds and cannot be distinguished based on this methodology. Results are reported as the pair. Relative retention time (RRT) shifts may occur, report cases that are within ±2% of average standard RRT as positive.
   (c) 3-methyl fentanyl may interfere with the quantitation of butyryl fentanyl/isobutyryl fentanyl by LC-MS/MS.
   (d) Meta-fluorofentanyl may interfere with the quantitation of para-fluorofentanyl and ortho-fluorofentanyl by LC-MS/MS.

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Chapter 16

Screening Analysis for Designer Stimulants by LC-MS/MS

Piotr Adamowicz and Bogdan Tokarczyk

Abstract

The increase in the number of new substances appearing on the drug market has been observed in the 1980s and 1990s of the last century, when many phenethylamine and tryptamine derivatives entered the market. However, the phenomenon of mass marketing of new designer stimulants (being the components of so-called "legal highs") began to develop since 2006 in Europe, and it was something new. Since then, the number of stimulants introduced on the drug market is growing regularly, rapidly, and intensively. Such a situation creates a need for comprehensive screening methods for detection of these drugs in biological specimens. The fast and simple liquid chromatography-tandem mass spectrometry qualitative screening procedure presented here is designed to detect and identify a wide range of designer stimulants in the blood. The assay has wide applicability for rapid screening of new stimulants in forensic or clinical samples. The procedure can be easily modified for additional novel psychoactive substances.

Key words Designer stimulants, New psychoactive substances (NPS), Legal highs, Drug screening, Blood analysis, LC-MS/MS

1 Introduction

In recent years, many designer stimulants have appeared on the drug market, and currently still the upward trend in the number of new drugs is continuing in many countries worldwide [1]. These substances are also known as "legal highs" or "research chemicals" and are sold mainly on the Internet. Officially, they are not intended for human consumption and are sold under trivial names, as collectibles, plant fertilizers, bath salts, room fresheners, or herbal incenses, in order to avoid criminal responsibility. Designer drugs are characterized by similar chemical structures to controlled substances. This is due to the fact that these substances are synthesized in order to circumvent the existing drug laws, usually by changing the structure of known illegal drugs. New designer stimulants are an important challenge for toxicologists. Screening of biological material for the presence of designer stimulants should be a part of routine procedures used in clinical and forensic laboratories.

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However, complex methods enabling the detection of such a wide spectrum of these compounds in biological materials are lacking. Constantly emerging drugs require that the methodology of their determination in biological material must be constantly updated. Unfortunately, immunochemical tests are not effective for diverse and variable new drugs. Analytical methods used in many laboratories such as gas chromatography coupled with mass spectrometry (GC-MS) or high-performance liquid chromatography with diode array detection (HPLC-DAD) are often insufficient. Due to insufficient sensitivity, the application of these methods is limited to acute or fatal poisonings.

Only modern coupled techniques, especially liquid chromatography with tandem mass spectrometry (LC-MS/MS), have the features that enable a comprehensive analysis of a wide range of designer stimulants in biological material [2–4]. In this context, we present a fast and simple LC-MS/MS screening procedure for the detection and identification of many popular designer stimulants in the blood in a single run. The method allows the screening of 80 designer stimulants using only 0.2 mL of the whole blood. The most important advantage of this method is that the procedure can be easily modified for more drugs. A similar procedure was used for the screening for 143 new psychoactive substances [5].

2 Materials

2.1 Laboratory Equipment

1. Liquid chromatograph coupled with mass detector (e.g., Agilent Technologies 1200 Series liquid chromatograph with 6460 Triple Quad mass spectrometer).
2. Agilent Technologies SB-C18 chromatography column (2.1 × 50 mm, 1.8 μm).
3. Thermoblock with evaporator.
4. Minicentrifuge for vials.
5. Vortexer.
6. Single-channel automatic pipettes.
7. Freezer for temperatures below −15 °C.
8. Refrigerator providing a temperature below 4 °C.

2.2 Reagents and Solutions

All solvents should be gradient grade or higher (*see* **Note 1**).

1. Mobile phase A: 0.1% formic acid in water (v/v). Add 1 mL of formic acid to 1 L of distilled water (or proportionately smaller volumes) (*see* **Note 2**).
2. Mobile phase B: 0.1% formic acid in acetonitrile (v/v). Add 1 mL of formic acid to 1 L of acetonitrile (or proportionately smaller volumes).

3. Working internal standard solution: 1 μg/mL mephedrone-D_3 in methanol. Add 10 μL of 0.1 mg/mL mephedrone-D_3 drug standard to 990 μL of methanol. Internal standard solutions must be stored below −15 °C, but not longer than 7 days (*see* **Note 3**).
4. Spiking controls solutions: 1 μg/mL selected stimulant(s) in methanol. Add 10 μL of each 1 mg/mL standard for the drug (s) of interest to 990 μL of methanol to obtain the concentration of 10 μg/mL. Dilute the obtained solution 10×, for example, add 100 μL to 900 μL of distilled water to obtain the final concentration of 1 μg/mL. These solutions must be stored below −15 °C, but not longer than 7 days (*see* **Note 3**).
5. Precipitation solution: iced acetonitrile (< −15 °C).

2.3 Supplies

1. 2 mL Eppendorf vials.
2. 2 mL glass vials with screw caps.
3. Polypropylene inserts (for autosampler vials).
4. Autosampler vials.
5. Graduated cylinder (0.5 L or 1 L).
6. Pipette tips.

3 Methods

3.1 Sample Preparation

1. Defrost unknown blood samples for approximately 30 min at room temperature.
2. Defrost drug-free blood for controls for 30 min at room temperature.
3. Pipette 0.2 mL of each unknown blood sample into a 2 mL Eppendorf vial. Pipette 0.2 mL of drug-free blood into a 2 mL vial for each control to be used.
4. Add 2 μL (final concentration 10 ng/mL) or 20 μL (final concentration 100 ng/mL) of each spiking control solution to the appropriate vial of drug-free blood, and vortex to mix (*see* **Note 4**).
5. Add 20 μL of working internal standard solution to all vials to obtain a final concentration of 100 ng/mL of mephedrone-D_3. Wait 10 min.
6. Precipitate the samples with iced acetonitrile (*see* **Note 5**). Add 600 μL of acetonitrile in 50 μL portions, vortexing the samples for 10 s after each addition. After adding the last portion of acetonitrile, mix the samples for 5 min, and centrifuge at 13,000 rpm (15,682 × *g*) for 5 min (*see* **Note 6**).
7. Transfer the organic solvent to a 2 mL glass vial.

8. Evaporate the acetonitrile to dryness under air or nitrogen at 30 °C (*see* **Note 7**).
9. Dissolve the dry residues in 100 μL of mobile phase A, and transfer to inserts for autosampler vials.

3.2 Chromatographic and Spectrometric Conditions

1. Column temperature: 20 °C. It is suggested to equip the column with an inline filter (4.6 mm, 0.2 μm) (*see* **Note 8**).
2. Mobile phase flow rate: 0.3 mL/min. Gradient conditions are shown in relation to mobile phase B content:

   0 min—10%

   6 min—100%

   7 min—10%

   14 min—10%
3. Injection volume: 10 μL.
4. Set the mass detector mode to positive ionization (ESI+) and dynamic multiple reaction monitoring (dMRM) (*see* **Note 9**).
5. Set the monitored MRM transitions, fragmentor voltages, and collision energies for individual compounds according to data presented in Table 1 (*see* **Note 10**). Example chromatograms are shown in Fig. 1.
6. Set the remaining mass detector parameters as follows: capillary voltage, 3000 V; gas flow (nitrogen), 11 L/min; and gas temperature, 325 °C; sheath gas flow, 11 L/min; sheath gas temperature, 325 °C; nebulizer pressure, 40 psi; and retention time window for all compounds, 1 min (*see* **Note 11**).

4 Notes

1. Store acetonitrile used for precipitation at temperatures below −15 °C. Store distilled water at temperatures below 4 °C. Store commercially purchased stock drug solutions at temperatures below −15 °C. Until analysis, keep blood samples at temperatures below −15 °C.
2. Keep mobile phase A in dark glass bottle to retard bacteria and algae growth.
3. Changes of standard concentration may occur in working solutions maintained over a prolonged period of time.
4. In order to control the sensitivity of the method as well as if the results are positive, the selected stimulants should be spiked into drug-free blood samples to prepare controls at concentrations of 10 and 100 ng/mL. Samples are initially run without drug-specific positive controls. The internal standard is used in the initial run as an indicator of successful extraction and

Table 1
List of designer stimulants covered by presented method with their names, mass spectrometer parameters, and retention times

Name	Precursor ion	Product ion	Fragmentor voltage [V]	Collision energy [V]	Retention time RT [min]	Relative retention time RRT
2-AI (2-aminoindane)	134.1	117 115 91.1	87	12 24 32	1.2	0.48
2-DPMP (desoxypipradrol)	252.2	167 91.1 65.1	120	16 24 60	5.4	2.16
2-FA/4-FA (2/4-fluoroamphetamine)	154.1	137 109 83	85	4 16 44	1.6	0.64
2,3-DMEC (2,3-dimethylethcathinone)	206.2	188.2 159.2 158.2	56	8 16 32	4.8	1.92
2,4-DMEC (2,4-dimethylethcathinone)	206.2	115.1 91.2 72.2	68	60 60 12	5.1	2.04
3,4-DMMC (3,4-dimethylmethcathinone)	192.1	174.1 159.1	85	8 20	5.03	2.01
3-CMC (3-chloromethcathinone)	198.1	180.1 145.1 144.1	25	8 16 32	2.54	1.02
4-CMC (4-chloromethcathinone)	198.1	180 145.1 144.1	83	8 16 36	2.54	1.02

(continued)

Table 1
(continued)

Name	Precursor ion	Product ion	Fragmentor voltage [V]	Collision energy [V]	Retention time RT [min]	Relative retention time RRT
3-EMC (3-ethylmethcathinone)	192.1	91.2 77.1 65.2	58	40 52 60	4.8	1.92
3-FMC (3-fluoromethcathinone)	182.1	164.1 149 148	93	8 20 36	1.3	0.52
3-FPM (3-fluorophenmetrazine)	196.1	135.1 115.1 109.1	25	20 32 24	2.2	0.88
3-MEC (3-methylethcathinone)	192.1	174.2 145.2 144.2	62	8 16 28	3.4	1.36
4-BMC (brephedrone, 4-bromomethcathinone)	242	145.1 144 77.1	85	12 36 60	3.6	1.44
4-CEC (4-chloroethcathinone)	212.1	194.1 144.1 77.1	86	8 28 60	4.23	1.69
4Cl-α-PVP (4-chloro-α-pyrrolidinovalerophenone)	266.1	125 111 74.1	102	24 48 132	5.47	2.19
4-EEC (4-ethylethcathinone)	206.2	188.2 159.1 144.1	25	8 16 28	4.98	1.99

4-EMC (4-ethylmethcathinone)	192.1	174.1 145.1 144.1	68	8 20 36	4.68	1.87
4-FMA (4-fluoromethamphetamine)	168.1	137.1 109 83.1	62	8 20 44	1.85	0.74
4-FMC (flephedrone, 4-fluoromethcathinone)	182.1	164.1 149 148	83	8 20 36	1.29	0.52
4-MBC (benzedrone)	254.2	236.1 91.1 65.1	87	8 24 60	5.55	2.22
4-MDMC (4-methyldimethcathinone)	192.1	91.2 77.2 72.2	54	40 60 28	2.9	1.16
4-MEAP (NEMNP, 4-methyl-α-ethylaminopentiophenone)	220.2	202.1 144.1 91.1	78	8 32 48	5.25	2.10
4-MEC (4-methylethcathinone)	192.1	174.1 145.1 91.1	143	8 16 36	3.34	1.34
4-MeMABP (4-methylbuphedrone)	192.1	174.2 145.2 144.2	56	8 20 32	4.4	1.76
4-MPD (4-methylpentedrone)	206.2	188.1 144.1 77.1	80	8 36 60	5.11	2.04
4-MPHP (PV-4, 4′-methyl-α-pyrrolidinohexiophenone)	260.2	140.1 105.1 91.1	104	24 20 48	5.72	2.29

(continued)

Table 1
(continued)

Name	Precursor ion	Product ion	Fragmentor voltage [V]	Collision energy [V]	Retention time RT [min]	Relative retention time RRT
4-MTA (4-methylthioamphetamine)	182.1	165 137 117	62	4 20 16	4.46	1.78
5-APB (5-(2-aminopropyl)benzofuran)	176.1	159.1 131.1 77	70	4 16 44	3.17	1.27
5-MAPB (5-(2-methylaminopropyl)benzofuran)	190.1	159.1 131 91.1	68	8 20 36	4.0	1.60
6-APB (6-(2-aminopropyl)benzofuran)	176.1	159.1 131 91.1	64	4 20 36	4.04	1.62
6-EAPB (1-(benzofuran-6-yl)-*N*-ethylpropan-2-amine)	204.1	159.2 131.1 91.1	25	8 20 36	4.7	1.88
6-IT (6-(2-aminopropyl)indole)	175.1	158.1 130 117	60	4 20 24	2.53	1.01
6-MAPB (6-(2-methylaminopropyl)benzofuran)	190.1	159.2 131.1 91.1	25	8 16 36	4.2	1.68
α-MT (α-methyltryptamine)	175.1	158.1 130 117	58	4 24 28	2.35	0.94

α-PVP (α-pyrrolidinopentiophenone)	232.2	126.1 91 77	85	24 24 48	4.56	1.82
α-PBP (α-pyrrolidinobutiophenone)	218.2	112.1 91.1 77.1	83	24 20 48	2.57	1.03
α-PPP (α-pyrrolidinopropiophenone)	204.1	133 105.1 98.1	120	16 24 28	1.65	0.66
α-PVT (α-pyrrolidinopentiothiophenone)	238.1	126.1 111 97	41	20 36 20	3.18	1.27
BDB (1,3-benzodioxolylbutanamine)	194.1	179.1 164.1 77.1	143	12 20 52	5.1	2.04
bk-DMBDB (dibutylone)	236.1	161 86.1 65.1	145	16 24 56	2.38	0.95
bk-MDDMA (dimethylone)	222.1	147 91.1 72.1	83	16 36 16	1.5	0.60
BMDP (4-methylenedioxy-*N*-benzylcathinone)	284.1	266.1 91 65.1	87	8 24 60	5.18	2.07
Buphedrone	178.1	160.1 91.1 77.1	85	4 16 48	1.83	0.73
Bupropion	240.1	184 166 131.1	93	4 12 24	5.05	2.02

(continued)

Table 1
(continued)

Name	Precursor ion	Product ion	Fragmentor voltage [V]	Collision energy [V]	Retention time RT [min]	Relative retention time RRT
Butylone	222.1	204.1 174.1 131	91	4 12 32	2.14	0.86
BZP (benzylpiperazine)	177.1	91.1 85.1 65.1	101	20 12 52	0.5	0.20
D2PM (diphenylprolinol)	254.2	236.1 165 130	83	8 56 28	4.91	1.96
Diethylpropion	206.2	105.1 100.1 77.1	112	16 20 48	1.88	0.75
Ephedrone	164.1	146 131 77	85	8 16 52	1.05	0.42
Ethylone	222.1	204.1 174.1 146.1	89	4 12 24	1.72	0.69
Ethylphenidate	248.2	84.1 56.1 55.1	85	20 56 56	5.1	2.04
Ethcathinone	178.1	160.1 131 117	89	8 16 28	1.31	0.52

Eutylone	236.1	218.1 188.1 174	89	8 16 32	2.97	1.19
MDPBP (3′,4′-methylenedioxy-α-pyrrolidinobutyrophenone)	262.2	161.1 112.1 65.1	118	16 24 60	3.6	1.44
MDPPP (3′,4′-methylenedioxy-α-pyrrolidinopropiophenone)	248.1	147 98.1 91.1	91	20 24 48	2.08	0.83
MDPV (methylenedioxypyrovalerone)	276.2	175.1 135 126.1	124	16 20 24	4.9	1.96
MeBP (methylbuphedrone)	191.2	105.1 79.1 77.1	99	20 40 52	0.74	0.30
MeOPP (para-methoxyphenylpiperazine)	193.1	150 120 65.1	116	16 36 60	2.1	0.84
Mephedrone/2-MMC/3-MMC	178.1	160.1 145.1 77.1	87	8 20 56	2.5	1.00
MePPP (4′-methyl-α-pyrrolidinopropiophenone)	218.2	119.2 98.2 91.2	54	24 28 40	4.1	1.64
Metamfepramone (dimethylcathinone)	178.1	133 105.1 72.1	85	12 20 24	1.2	0.48
Methedrone (4-methoxymethcathinone)	194.1	176.1 161 146	85	8 16 28	1.8	0.72

(continued)

Table 1
(continued)

Name	Precursor ion	Product ion	Fragmentor voltage [V]	Collision energy [V]	Retention time RT [min]	Relative retention time RRT
Mexedrone	208.1	158.2 119.1 91.2	56	8 20 40	3.3	1.32
MDMC (methylone)	208.1	190 160 132	91	4 12 24	1.56	0.62
MPA (methiopropamine)	156.1	125 97 58.2	58	8 20 4	1.0	0.40
MPBP (4′-methyl-α-pyrrolidinobutiophenone)	232.2	161.1 105.1 91.1	85	12 24 48	4.79	1.92
MPHP (4′-methyl-α-pyrrolidinohexiophenone)	260.2	140.2 105.1 91.1	25	28 24 52	5.9	2.36
MPPP (desmethylprodine)	248.2	174.1 44.2 42.2	97	8 20 60	4.61	1.84
Naphyrone	282.2	211.1 155 141	126	12 24 20	5.76	2.30
NEB (*N*-ethylbuphedrone)	192.1	174.1 130 91.1	68	8 32 28	2.25	0.90

NEH (*N*-ethylhexedrone)	220.2	130.1 91.1 77.1	84	36 32 60	5.2	2.08
N-ethylpentylone (ephylone)	250.1	232.1 202.2 174.1	84	8 16 32	4.7	1.88
Pentedrone (α-methylaminovalerophenone)	192.1	174.1 132.1 91.1	89	8 16 24	3.91	1.56
Pentylone (methylenedioxypentedrone)	236.1	218.1 188.1 175.1	62	8 16 20	4.4	1.76
PV-7 (α-PHP, α-pyrrolidinohexiophenone)	246.2	140.2 91.2 77.2	54	24 24 60	5.3	2.12
PV-8 (α-PHPP)	260.2	154.2 91.1 77.1	5	28 24 60	5.62	2.25
TFMPP (trifluoromethylphenylpiperazine)	231.1	188 145 44.1	122	16 44 20	5.08	2.03
Mephedrone-D3 (internal standard)	181.1	163.1	87	8	2.5	1.00

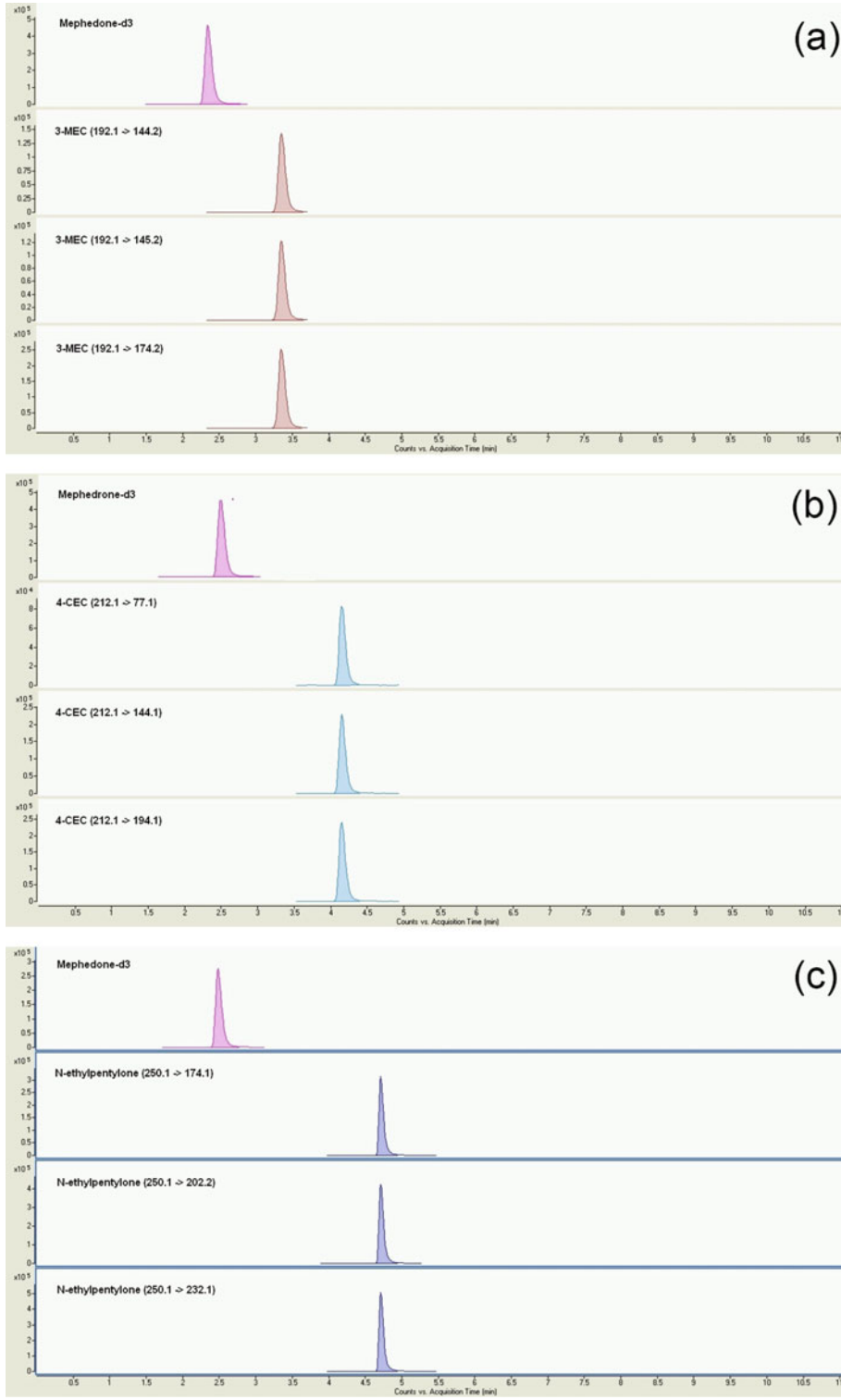

Fig. 1 Example of chromatograms obtained for blood from real forensic cases in which 3-methylethcathinone (**a**), 4-chloroethcathinone (**b**), and *N*-ethylpentylone (**c**) were revealed. Subsequent analysis by other quantitative methods determined drug concentrations to be, respectively, 3-MEC, 69 ng/mL; 4-CEC, 49 ng/mL; and *N*-ethylpentylone, 196 ng/mL

analysis, based on acceptable intensity and retention time. Samples that meet internal standard criteria and demonstrate the presence of another compound(s) in Table 1 are repeated, alongside drug-free blood spiked with standards of the appropriate drug or drugs.

5. We find that the use of iced acetonitrile (stored at temperatures below −15 °C) provides better isolation efficiency.
6. Never add the entire volume of acetonitrile at once because it causes immediate protein precipitation, which significantly reduces the yield of the process.
7. In this form, the samples may be frozen at temperatures below −15 °C and stored up to 1 month. Do not evaporate at higher temperatures. Some compounds (especially cathinones) might be lost.
8. The column used tends to clog up during biological material analysis. The use of interchangeable filters significantly increases the life of the column.
9. The application of dynamic MRM provides an enhanced sensitivity (due to increased number points per peak) by utilizing the retention time window of each analyte [6].
10. Monitor three MRM pairs for each compound. This ensures the specificity of the method. The procedure is open, meaning that the procedure can be easily extended for additional new designer drugs. The most intense MRM transitions for substances not listed here can be easily obtained with the use of the Agilent Mass Hunter Optimizer. This software automatically selects four of the most abundant fragment ions for defined precursor ion and optimizes fragmentor voltage and the corresponding collision energy for each transition.
11. Interpretation of results: Concentrations are estimated using the spiked controls. Samples for which the estimated concentration (based on comparison with the controls) is higher than the cutoff of 5 ng/mL are considered as presumptive positive and subjected to subsequent targeted quantitative analyses (not part of this procedure).

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Chapter 17

Drug Screening Using Liquid Chromatography Quadrupole Time-of-Flight (LC-QqTOF) Mass Spectrometry

Jennifer M. Colby and Kara L. Lynch

Abstract

Drug screening using high-resolution mass spectrometers, including quadrupole time-of-flight mass analyzers (QqTOFs), is becoming increasingly popular due to the additional flexibility that these instruments offer laboratories. Liquid chromatography (LC) coupled to TOF, as in an LC-QqTOF, offers comparable sensitivity and a shortened method development time relative to triple quadrupole-based mass spectrometry. In addition, LC-QqTOF data that are collected in untargeted mode can be analyzed retrospectively to detect additional compounds that were not predefined targets. Much of the power of LC-QqTOF lies in data processing, and the data analysis workflow that a lab uses must be adequately validated.

Key words High-resolution mass spectrometry, Time of flight, Drug screen, Toxicology

1 Introduction

High-resolution mass spectrometry (HRMS) using quadrupole time-of-flight (QqTOF) or Orbitrap technology has gained recognition as a valuable tool for broad-spectrum drug screening in a variety of biological matrices. In contrast to triple quadrupole-based liquid chromatography tandem mass spectrometry (LC-MS/MS) methods, which collect nominal mass data primarily in a targeted manner, HRMS instruments collect untargeted, accurate mass data for precursor and product ions. Similar to LC-MS/MS methods, compounds can be identified by targeted data analysis (i.e., library searching) in which the precursor mass, retention time, and product ion spectrum are compared to that of a reference standard. It has been demonstrated that the detection capabilities of LC-QqTOF for drug screening using targeted data analysis are comparable to LC-MS/MS [1]. In addition to targeted analysis, other data analysis strategies are possible (e.g., suspect and untargeted screening) [2–4]. These additional strategies are particularly attractive in the setting of drug screening, as they allow for the

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identification of compounds that are not included in the library or are unexpected. As a result, HRMS permits identification of both expected and unexpected compounds in a single analytical run. Tentative identification of unknown compounds can be made in the absence of a reference standard or a library spectrum. Once a compound is tentatively identified, an analytical standard can be purchased and tested to confirm that the analytical retention time and fragmentation pattern match that observed in the test sample.

The illicit drug market is constantly evolving, making it difficult for analytical toxicology laboratories to maintain methods capable of detecting all potential toxins and emerging drugs. Since data are acquired in an untargeted manner with HRMS, they can be retrospectively analyzed for new and emerging novel psychoactive substances (NPS) and synthetic designer drugs, adding to the appeal of this technology in the clinical and forensic setting. The addition of emerging drugs to HRMS methods using targeted data analysis only requires the purchase of an analytical standard to establish the retention time and acquire a high-resolution mass spectrum for addition to the spectral library used by the laboratory. Subsequently the limit of detection, matrix effects, and other validation parameters can be evaluated following each laboratory's protocol. HRMS has clearly emerged as the predominant method for the detection of NPS; however, this technology is not readily available in most routine clinical and forensic laboratories. This chapter will describe an HRMS method using an LC-QqTOF instrument for drug screening. It will primarily focus on the use of targeted data analysis for drug screening. This method can be adapted to include any pharmaceutical or illicit drug with the purchase of an analytical standard. Validation of the analytical method is further described in Thoren et al. (2016), and validation of the data analysis parameters is described in Colby et al. [5].

2 Materials

2.1 Standards and Reagents

1. 50:50 methanol/acetonitrile: Mix 15 mL of methanol with 15 mL of acetonitrile (*see* **Note 1**). Solution is stable at 15–30 °C for 1 year when stored in glass bottles (e.g., Pyrex).
2. Specimen preparation buffer: Mix 25 mL of 50:50 methanol/acetonitrile with 175 mL of water to prepare 200 mL of preparation buffer. Solution is stable at 15–30 °C for 1 year when stored in glass bottles (e.g., Pyrex).
3. 1 M ammonium formate: Measure 6.305 g of ammonium formate using an analytical balance. Add the ammonium formate to a 100 mL volumetric flask, and fill to 100 mL with water. Solution is stable at 15–30 °C for 1 year when stored in glass bottles (e.g., Pyrex).

4. Mobile phase A: 5 mM ammonium formate and 0.05% formic acid in water. Add 10 mL of 1 M ammonium formate, 1 L of water, and 1 mL of formic acid to a graduated cylinder, and then add water to bring the volume to 2 L. Solution is stable at 15–30 °C for 1 month when stored in glass bottles (e.g., Pyrex).
5. Mobile phase B: 0.05% formic acid in 50:50 methanol/acetonitrile. Mix 1 L of acetonitrile with 1 L of methanol in a 2 L graduated cylinder. Using a volumetric pipette, remove 1 mL of the mix, and then add 1 mL of formic acid. Solution is stable at 15–30 °C for 1 month when stored in glass bottles (e.g., Pyrex).
6. Pump wash solution: 50:50 water/methanol. Mix 1 L of water with 1 L of methanol in a 2 L graduated cylinder. Solution is stable at 15–30 °C for 6 months when stored in glass bottles (e.g., Pyrex).
7. Quality control mix: 1 μg/mL each of 20–25 drugs in 50:50 methanol/acetonitrile (*see* **Notes 2** and **3**). Add 5 mL of 50:50 methanol/acetonitrile to a 10 mL volumetric flask. Add 10 μL of each 1 mg/mL drug reference standard to be included. Fill to volume with 50:50 methanol/acetonitrile, and mix well. Prepare 1 mL aliquots in glass vials with PTFE-lined caps. Example mixes are shown in Table 1. Mixes are stable at −20 °C for 1 year, or until the stock standard solutions expire, whichever comes first.
8. Fentanyl-D5 internal standard working solution: 1 μg/mL fentanyl-D5 in specimen preparation buffer. Add 100 μL of fentanyl-D5 stock standard (100 μg/mL) to 9.9 mL of specimen preparation buffer. Prepare 1 mL aliquots in glass vials with PTFE-lined caps. Stable for 1 year at −20 °C, or until the stock solution expires, whichever comes first.
9. Serum/plasma protein dump solution: 200 ng/mL fentanyl-D5 in acetonitrile. Add 500 μL of fentanyl-D5 stock standard (100 μg/mL) to a 250 mL volumetric flask, and fill with acetonitrile. Prepare 10 mL aliquots in glass vials or bottles. Stable for 1 year at −20 °C, or until the stock solution expires, whichever comes first.
10. Negative control: Drug-free human urine or serum. Stable at 4 °C until expiration date on label (*see* **Note 4**).
11. Positive controls: 100 ng/mL of each quality control mix. Add 1 mL of each quality control mix into its own 10 mL volumetric flask. Fill to volume with drug-free human urine or serum (match the matrix of the samples to be tested). Depending on the drugs that it contains, the positive control mix may be stable at 4 °C for up to 30 days or up to 1 year at −80 °C. Prior to freezing, prepare aliquots with a volume that can be used within the refrigerated stability window of the mix (*see* **Note 5**).

Table 1
Example of drug mixes

Mix A	Mix B	Mix C
2C-T2	6-APB/5-APB	Delta9-THC
4-MePPP	7-Aminoclonazepam	2-Hydroxyethylflurazepam
4-MTA	7-Aminonitrazepam	6-monoacetylmorphine
Amoxapine	Aripiprazole	9-hydroxyrisperidone
Atropine	Brompheniramine maleate	Acetaminophen
Cannabidiol	Buprenorphine glucuronide	Alpha-hydroxytriazolam
Chlorpheniramine	Cannabinol	Diazepam
Clomipramine	Cathinone	Droperidol
Clonazepam	Codeine	Gabapentin
Desipramine	Desalkylflurazepam	Hydrocodone
Dihydrocodeine	Diphenoxylate	Levorphanol
Diphenhydramine	Flunitrazepam	Loxapine
DOM	Hydroxyzine	Methadone
Doxepin	Meperidine	Methylphenidate
Ecgonine methyl ester	Meprobamate	Mianserin HCl
Fluoroamphetamine	Olanzapine	N-Desmethylclomipramine
Lorazepam	Oxcarbazepine	Nicotine
MBDB	Phentermine	Nordoxepin
Methocarbamol	Quetiapine	Norketamine
Oxycodone	Zaleplon	Venlafaxine

4-MePPP 4′-methyl-α-pyrrolidinopropiophenone, *4-MTA* 4-methylthioamphetamine, *DOM* 2,5-dimethoxy-4-methylamphetamine, *MDBD* methylbenzodioxolylbutanamine, *APB* 2-aminopropylbenzofuran, *THC* tetrahydrocannabinol, *HCl* hydrochloride

2.2 Supplies

1. 12 × 75 mm plastic test tubes.
2. 13 × 100 mm glass tubes (*see* **Note 6**).
3. 2 mL autosampler vials and caps with polytetrafluoroethylene (PTFE)-lined septa.
4. Amber vials with PTFE-lined caps for drug and internal standard solutions.
5. Volumetric glassware (pipettes and flasks, Class A).
6. Graduated cylinder (2 L).
7. Glass bottles to store reagents.

8. Plastic transfer pipettes (*see* **Note 7**).
9. UPLC columns (Kinetex C18, 2.6 μm, 3 × 50 mm) and guard columns (SecurityGuard ULTRA cartridges, C18) or similar.
10. APCI calibration solution for 5600 TripleTOF®.

2.3 Equipment

1. Benchtop centrifuge (Eppendorf Microcentrifuge 5430, or similar).
2. Sample concentrator/evaporator (TurboVap LV evaporator, or similar).
3. Air displacement pipettes (Eppendorf Reference 2 Pipettes, or similar).
4. Vortexer.
5. Centrifuge (Eppendorf 5810 or similar).
6. An HPLC system compatible with ABSciex Analyst software (e.g., Shimadzu LC-20ADXR Prominence) with degasser, binary pump, solvent-switching valve, temperature-controlled autosampler, and temperature-controlled column compartment.
7. Nitrogen generator or liquid N_2 dewar capable of supplying curtain, collision, and source gases.
8. 5600 TripleTOF® quadrupole time-of-flight mass spectrometer with a DuoSpray™ source and automatic calibrant delivery system.
9. Analyst® 1.5, PeakView® 2.0, and MasterView™ 1.0 software.

3 Methods

3.1 Analysis of Urine

1. Aliquot approximately 1 mL of each patient urine specimen into a 12 × 75 mm plastic test tube using a disposable, plastic transfer pipet. Cap the tube.
2. Centrifuge all patient urine specimens for 10 min at 450 × *g* in the centrifuge.
3. Label an amber autosampler vial for the negative control, positive control, and each patient urine. Due to the possibility of carryover, a double blank (no drug, no internal standard) sample must be injected between each patient sample. Each double blank can be injected up to five times before being replaced with a fresh vial.
4. Pipet 1000 μL of sample preparation buffer into each double blank vial.
5. Pipet 700 μL of sample preparation buffer into each control and patient vial.

6. Pipet 100 μL of internal standard into each vial, except for the double blank.
7. Pipet 200 μL of negative control, positive control, and patient urine to the correspondingly labeled vials.
8. Cap the vials and vortex to mix.
9. Place the vials in the autosampler tray for testing.

3.2 Analysis of Serum/Plasma

1. Aliquot 250 μL of each serum or sodium heparin plasma specimen or control sample using an air displacement pipette with disposable tips into a 1.5 mL microcentrifuge tube. Add 750 μL of protein dump solution. Pipette up and down to mix. Cap the tube. Vortex for at least 30 s.
2. Centrifuge specimens for 10 min at 8500 × *g* in a benchtop centrifuge.
3. Carefully remove samples from the centrifuge, ensuring that the pellet at the bottom of the tube is not disturbed.
4. Open the tube, and transfer 750 μL of supernatant into a 13 × 100 mm glass tube. Some supernatant should remain in the tube.
5. Dry supernatant under nitrogen flow at 37 °C for 20 min or until dry.
6. Label an amber autosampler vial for the negative control, positive control, each patient specimen, and double blank samples. Due to the possibility of carryover, a double blank (no drug, no internal standard) sample must be injected between each patient sample.
7. Transfer 1000 μL of sample preparation buffer to the double blank vial. Cap and place in the autosampler tray.
8. Pipet 100 μL of sample preparation buffer into the tubes containing dried control and patient samples, ensuring that the dried sample is resuspended.
9. Pipet the resuspended patient and control samples into the appropriate autosampler vials.
10. Cap the vials and place in the autosampler tray for testing.

3.3 Instrument Operating Conditions

Liquid chromatography system:

1. Injection volume: 10 μL
2. Flow rate: 400 μL/min
3. LC parameters:

   0 min: 2% mobile phase B (MPB)

   10 min: 98% MPB

   Wash 2 min at 100% MPB

Re-equilibrate 2 min at 2% MPB

5600 QTOF mass spectrometer:

4. Ion source: positive electrospray, 500 °C, ion spray voltage floating 5500 V, declustering potential 100 V
5. Gas settings: source gas 1–30 PSI, source gas 2–30 PSI, curtain gas 25 PSI
6. Ion release delay: 67
7. Ion release width: 25
8. Full-scan TOF-MS from 50 to 700 Da
9. Information-dependent acquisition of product ion spectra for ≤20 candidate ions per cycle
10. Automatic calibration verification of TOF and MS/MS mass accuracy every five injections

3.4 Data Analysis

1. Load the data file(s) and premade targeted analysis extraction ion chromatogram (XIC, Fig. 1) list into MasterView (*see* **Notes 8** and **9**).

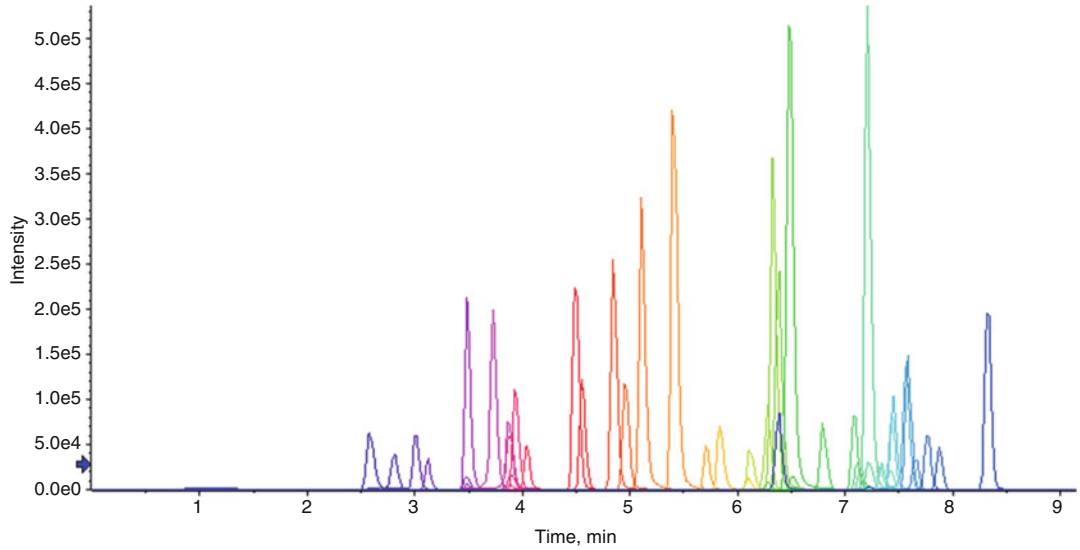

Fig. 1 Extracted-ion chromatogram of a mix of 40 drugs and metabolites. Drug (retention time in minutes): morphine (2.59), oxymorphone (2.81), hydromorphone (3.01), codeine (3.49), amphetamine (3.69), oxycodone (3.73), 6-monoacetylmorphine (3.88), hydrocodone (3.88), MDA (3.89), methamphetamine (3.90), desmethyltramadol (3.94), MDMA (4.05), benzoylecgonine (4.5), norfentanyl (4.56), tramadol (4.85), 7-aminoclonazepam (4.96), cocaine (5.11), norbuprenorphine (5.71), fentanyl (6.12), fentanyl-D5 (6.12), flurazepam (6.33), midazolam (6.38), EDDP (6.49), buprenorphine (6.51), clonazepam (6.73), alpha-hydroxymidazolam (6.80), alpha-hydroxytriazolam (7.12), nitrazepam (7.15), methadone (7.21), alpha-hydroxyalprazolam (7.22), oxazepam (7.33), lorazepam (7.43), flunitrazepam (7.44), 2-hydroxyethylflurazepam (7.45), alprazolam (7.57), triazolam (7.58), desalkylflurazepam (7.66), temazepam (7.77), nordiazepam (7.88), diazepam (8.33)

2. Ensure that ion extraction settings display precursor mass search of ±30 ppm, a retention time window of ±15 s, and a minimum peak intensity of 1 count per second.
3. Verify that a combined score threshold of >70 and combined scoring algorithm using 10% mass error, 10% retention time error, 10% isotope error, and 70% library match have been programmed as positivity criteria.
4. Process data.
5. Review presumptive positive extracted ion chromatograms for visual assessment of peak shape and library match (*see* **Note 10**).

4 Notes

1. All reagents, including water and solvents, must be analytical grade or better. Glassware used to prepare reagents should be free of detergents or other residues. Dedicated glassware used only for LC-MS reagent preparation is ideal. For measurement purposes, Class A volumetric glassware should be used whenever possible. Though they are not critical, positive displacement pipettes with disposable tips may be useful in measuring small volumes of solvent (e.g., for measuring drug standards).
2. Once opened, drug standards should be transferred to amber colored vapor-tight vials (PTFE-lined caps) for storage at −20 °C or −80 °C. Wrapping the top of the sealed vial with a narrow strip of Parafilm can help preserve standards for future use. Using amber vials helps protect drugs that degrade in light.
3. Each mix should contain different drugs. Drugs should not be grouped into mixes by class; instead, each mix should contain drugs with varying retention times and physical/chemical properties. The mixes of drug standards may be used in method development and validation, as well as to spike positive QC samples. Spiked samples can be used to establish retention time, product ion spectrum, and to verify lower limit of detection.
4. Each new lot of drug-free human serum or urine should be tested in-house to verify that it is truly free of all dugs. Most commercial laboratories that prepare human-derived materials for sale do not test for every drug and, in particular, do not test for a range of novel psychoactive substances. It is far less costly to determine up front whether a lot is suitable for use than to have to discard contaminated quality control materials.
5. The goal of quality control is to verify instrument performance across all drug categories and to confirm the chromatographic

separation meets expectations. The method is tested by injection of negative and positive control samples. To prevent wastage of reagents and analytical time, the positive control sample contains only a subset of the 100+ compounds in the method. The drugs are chosen based on prevalence in the population, chromatographic retention time, and presence of isomers. The presence/absence of each compound, peak area, and retention time are recorded for the drugs present in the positive control. These parameters are tracked over time to demonstrate stability of the method.

6. The wider the diameter of the glass tube that is used for evaporating samples, the faster they will dry. The choice of what tube to use depends on what tubes will fit in the drying apparatus that is in use in the laboratory.
7. The use of plastic transfer pipettes should be undertaken with caution. Compounds may be more susceptible to adsorb to some brands of pipettes than others. Once a suitable pipette is identified, introduction of a new supplier of pipettes should be avoided. If this is unavoidable, adsorption can be tested using spiked samples.
8. One of the rationales for collecting untargeted, full scan, HRMS data is that different data analysis techniques can be used to maximize the information gleaned from each sample. The data analysis workflow that is of greatest utility in routine analysis is known as targeted analysis. To perform targeted analysis, the analyst must know the accurate mass, chromatographic retention time, isotope pattern, and product ion spectrum of the targeted compounds. Accurate mass and isotope pattern can be predicted based on the empirical formula of the compound of interest. Retention times must be established on the LC-MS system where the method will be performed. It is ideal to collect product ion spectra on the LC-MS where the method will be run, using a dedicated product ion scan. The spectrum can be added to the in-house compound library. Retention times are typically established by averaging the observed retention time from $\geq$3 injections on $\geq$2 individual columns. Targeted data analysis methods may include any number of compounds. Including additional compounds is simple, compared to a traditional targeted acquisition, because compound-dependent parameters do not need to be developed. If the retention time is established and the product ion spectrum is included in the library, the compound can be detected using the targeted compound list. Additional validation experiments (e.g., recovery, lower limit of detection, interference testing, etc.) are necessary when adding a new compound to the method, but these are not typically too labor intensive.

9. After the parameters of interest have been established, a targeted compound list is built in MasterView. Positivity is assessed using combined scores. Additional details on how to establish and validate data analysis parameters can be found in Colby, Thoren, and Lynch, *Journal of Analytical Toxicology*, 2017 Jan; 41(1): 1–5.
10. Compounds identified by the targeted data analysis approach may be reported as positive or as presumptive positive, depending on the laboratory's reporting criteria.

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Chapter 18

Alternate Matrices: Meconium, Cord Tissue, Hair, and Oral Fluid

Kendra L. Palmer and Matthew D. Krasowski

Abstract

Drug testing commonly involves serum, blood, or urine. More recently, alternative specimens for drug testing have been increasingly used for clinical and forensic toxicology. Examples include oral fluid (saliva), hair, meconium, and umbilical cord tissue. Each of these matrices has unique properties that provide advantages for certain applications. Oral fluid has easier and less invasive collection requirements than urine, the most common specimen for drug screening. Oral fluid drug testing is common in Europe and steadily gaining popularity in the United States. Hair accumulates drugs and drug metabolites and provides a much longer window of detection than blood or urine. Meconium and umbilical cord tissue each allow for assessment of prenatal drug exposure over the course of months. Limitations of these alternative matrices include need for laboratory-developed tests (exception being some oral fluid immunoassays), challenges with the specimen matrix, and incomplete understanding of drug incorporation and kinetics. This chapter briefly describes each of the above alternative specimens in terms of their utility, advantages, and limitations.

Key words Drug screening, Neonatal testing, In utero exposure, Saliva testing, Detection window

1 Introduction

Historically, blood and urine were the mainstay specimens for drug testing; however, the use of alternative specimens in drug testing has become increasingly important in clinical and forensic toxicology. Some commonly used alternative specimens include oral fluid, hair, meconium, and umbilical cord tissue. Each of these matrices has unique qualities which provide advantages in various settings. This chapter will briefly describe each of the above alternative specimens in terms of their utility, advantages, and limitations. Detailed testing methods will be presented in subsequent chapters.

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2 Oral Fluid

Oral fluid is the liquid present in the oral cavity and consists primarily of saliva which is an aqueous solution produced by the salivary glands. Other minor constituents include oral mucosal transudate, bacteria, and epithelial cells. Oral fluid, as an alternative to blood and/or urine, is becoming a more widely used matrix. While oral fluid is commonly used in Europe, its use in the United States is still limited, and oral fluid testing is not widely available on routine chemistry analyzers. In areas where it is utilized, oral fluid is predominantly used in workplace testing and drug monitoring in substance abuse programs [1]. Oral fluid has some advantages over urine, which is another specimen often used in these settings. The primary advantage to oral fluid testing is that collection is simple and noninvasive, which eliminates the need for restroom facilities and makes it possible to collect in remote settings. Oral fluid collection can be more closely observed than urine collection, which limits the opportunity for specimen adulteration and also the need for same-sex collectors. An interesting feature of oral fluid as a toxicology specimen is that the parent drug is frequently the predominant substance [2]. For example, following use of heroin, oral fluid may contain the parent drug itself in addition to the metabolite 6-monoacetylmorphine (6-MAM). In contrast, heroin itself is rarely detected in blood or urine; the metabolites 6-MAM and morphine are much more common in these specimens.

There are some limitations to oral fluid testing. Drug concentrations are typically lower than those found in urine. Also, collecting sufficient sample volume to allow for confirmatory testing, should it be necessary, can be difficult. This is especially problematic as some drugs, including anti-adrenergic and anticholinergic drugs, will result in reduced salivation [3]. The oral fluid can be contaminated from ingested food or beverages, and drug concentrations will be higher for drugs that are smoked, inhaled, insufflated, or taken orally. When testing in a quantitative manner, this may be a limitation as concentrations will not correlate well with blood concentrations. However, when performing qualitative testing, this may present as an advantage.

Due to the relative newness of this specimen type, analyte stability has not been well characterized. Similarly, collection device designs are still being perfected. Some early sample collection devices caused adsorption of the drug to the device, resulting in false-negative test results.

3 Hair

Toxicology testing on hair, in various forms, has occurred since the mid-1800s. Although limited to heavy metal detection for the first century, advances in detection methods now allow for detection of a wide array of drugs and their metabolites. Hair testing is often used in conjunction with criminal and forensic investigations, including in drug-related deaths, child protection, and drug-facilitated crime. Hair testing has also become a component of drug monitoring in rehabilitation programs, in workplace drug testing, and for regranting of drivers' licenses [4].

Hair is a unique matrix for toxicology testing, in that it is able to provide historical data of drug use and can provide a very large detection window [5–8]. Once incorporated into the hair, drugs remain relatively stable and can theoretically be detected for up to several years, although detection windows of several months are more likely in practical terms [9]. This provides a detection window that is substantially longer than other matrices such as blood or urine, which can only detect use from hours to weeks, depending on the substance. The longer window of detection can be especially useful in assessing long-term abstinence. Hair toxicology testing may also provide some utility in assessing cases of possible child abuse or any other case when there is a delay in medical presentation [10]. In settings where a remote but narrow time frame is of interest, great caution must be used in result interpretation as the long window of detection can lead to misinterpretation.

Depending on the processing protocol, hair toxicology testing may also be able to provide information on environmental contamination. In some settings, such as child abuse assessment, the environmental exposure to illicit substances may be of interest. However, the potential of environmental contamination can introduce some uncertainty.

One distinct advantage of hair toxicology testing is that samples may be stored for long periods of time without the need for refrigeration. As with any specimen type, hair as a matrix for toxicology testing also has some limitations. Hair is a difficult matrix to analyze, and outside of reference laboratories, few clinical laboratories have the capability to perform this type of testing. An additional limitation of hair testing is that some individuals lack a sufficient amount of hair to facilitate testing—this is especially problematic with young children and infants and limits utility in newborn drug screening. Additionally, some individuals may remove their hair in an attempt to avoid testing.

Finally, the mechanisms of drug incorporation into hair are not completely understood, and evidence suggests that there may be some variability in drug binding which may be based on molecular size and structure, pH, and lipid solubility [11]. It has also been

suggested that melanin content of hair may influence the incorporation process, which may result in bias depending on hair color. Although drugs incorporated into hair are fairly stable under normal conditions, they can undergo decomposition when exposed to excessive ultraviolet radiation or bleaching. Once incorporated into the growing hair, it takes 7–10 days before the hair containing drug reaches the surface and then some additional time before the hair is long enough to sample. While this delay is not necessarily a limitation, it is a characteristic of hair toxicology testing which must be understood. There are a sufficient number of complexities to hair toxicology testing that an understanding of hair anatomy and physiology is required for proper interpretation of results.

4 Meconium

Meconium is the earliest stool which is formed by the fetus. Formation begins around the 12th week of gestation and continues to accumulate until it is passed shortly after delivery. The vast majority of healthy, full-term newborns pass their meconium within the first 48 h after birth [12]. The meconium is composed of intestinal epithelial cells, mucus, and bile, as well as substances ingested by the fetus in utero, including amniotic fluid and lanugo.

For some time, meconium was the gold standard specimen for detection of fetal drug exposure, and it is still widely used at many institutions [13, 14]. It also remains an important specimen when other sample types, such as umbilical cord tissue, are not available. One characteristic which has made meconium a popular specimen for newborn drug screening is its large window of detection. Because meconium accumulates during the entire second and third trimester, it should theoretically contain any drug that the fetus was exposed to during that time. In practice, detection of third trimester drug exposure may be more sensitive than isolated second trimester exposure, due to dilution and drug degradation over time [15]. Even with the possibility of false negatives early in the second trimester, meconium testing provides a much larger window of detection than other specimens, such as urine, which may only provide evidence of exposure during the days preceding delivery [13, 14].

The use of meconium for newborn drug screening also presents some challenges [13, 14, 16, 17]. There are several pre-analytic issues which can complicate meconium testing. Infants that are post-term or that experience stress in utero may pass their meconium prior to delivery, which makes it unavailable for collection after birth. Similarly, infants born prematurely may have delayed passage of meconium (on average, 7.8 days for very premature infants) due to ongoing maturation of intestinal function and resulting intestinal hypomotility [18]. Meconium passage may be

even further delayed in infants receiving morphine therapy. In practice, this may lead to missed collections due to expired or forgotten collection orders. Additionally, the risk factors used to determine if drug screening is indicated may not be immediately apparent and may not be discovered until after the meconium has been discarded.

The stability of drugs and drug metabolites is also variable in meconium, with particularly low stability of 6-MAM (heroin metabolite) in meconium at a variety of temperatures (including refrigerated, room temperature, and body temperature) [19]. Low stability was also observed for 7-aminoclonazepam (major metabolite of the benzodiazepine clonazepam) and chlordiazepoxide [19].

Many infants share a hospital room with their mother and can have several family members involved in their care. As a result, meconium samples can be accidentally or intentionally disposed of by family members. Meconium can also present some analytic challenges as well. Meconium is a sticky and heterogeneous matrix which makes it a challenging specimen. As a result, meconium testing is performed by very few laboratories. Lastly, there are some challenges in result interpretation. Any medication given to the newborn, prior to meconium passage, will also be detected. This is especially problematic with therapeutic opioids which will be indistinguishable from maternal opioid abuse during pregnancy.

5 Umbilical Cord Tissue

A newer, alternative matrix which can also be used for newborn drug testing is umbilical cord tissue. Studies have shown a high rate of concordance between drug detection from meconium and cord tissue samples. Testing methods for cord tissue were developed in 2006, and since that time, it has been gaining popularity due to some distinct advantages over meconium testing [20–24]. Similar to meconium testing, cord tissue has a large window of detection and should contain drugs that the fetus was exposed to during the third and possibly second trimesters [14, 21]. Drugs incorporated into umbilical cord tissue early in pregnancy are subject to degradation over time, as with drugs in meconium. Unlike meconium, cord tissue can be collected at the time of delivery. This solves many of the sample collection issues that complicate meconium testing, including premature and delayed passage, discarded samples, and potential sample tampering. One approach taken by hospitals which have instituted cord tissue testing involves collecting umbilical cord samples from every delivery and storing the samples for several weeks until clinical decisions regarding newborn drug testing have been made [25]. Cord tissue specimens can be stored for several weeks or more at refrigerator temperature. Collecting samples at the time of delivery also avoids detection of medications

given to the newborn after delivery, which greatly simplifies result interpretation.

Umbilical cord tissue testing is not without its own limitations. Cord tissue may not be available for infants who are transferred from an outside institution after delivery. Similar to other alternative matrices, umbilical cord tissue is a difficult matrix to test, and as a result, few laboratories perform testing. It has been found that cord tissue testing will detect some medications administered to the mother during delivery. This is most likely due to contamination from cord blood. There is not a clear understanding of whether passive exposure to marijuana or other drugs could result in a positive result. This combined with various factors affecting drug detection can make result interpretation difficult. Lastly, detailed studies of analyte stability in umbilical cord tissue, as have been done in meconium [19], have yet to be reported.

6 Summary

While each of these alternative specimens cannot be used as a perfect replacement for blood or urine, each alternative matrix has unique characteristics which can provide advantages when utilized in the appropriate settings. Oral fluid can be useful where a noninvasive method to detect recent substance use is needed. Hair testing is an ideal specimen when historical information of drug use is of interest. Meconium and cord tissue have similar utility in newborn drug screening where a large window of detection is desired.

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Chapter 19

Salt-Assisted Liquid-Liquid Extraction of Meconium for Analysis of Cocaine and Amphetamines by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Meconium, the first stool of a newborn, can be analyzed to identify prenatal exposure to drugs of abuse. Meconium accumulates in a fetus during the second and third trimesters of pregnancy providing a wide window of exposure. Identification of in utero drug exposure is essential for the diagnosis and treatment of infants for dependency/withdrawal caused from the exposure. However, testing of meconium samples is often cumbersome and time-consuming. Unlike liquid samples, meconium is a viscous, semisolid, tar-like substance that needs to be individually weighed prior to extraction. Additionally, the meconium matrix is not homogeneous and not easily mixed or extracted. A method for analyzing cocaine and metabolites as well as amphetamines in meconium utilizing ceramic homogenizers prior to salt-assisted liquid-liquid extraction and liquid chromatography tandem-mass spectrometry (LC-MS/MS) is presented.

Key words Meconium, Ceramic homogenizers, SALLE, Cocaine, Amphetamine

1 Introduction

In a recent survey, 4.7% of pregnant women aged 15–44 reported using illicit drugs during the previous month [1]. In utero drug exposure can cause several developmental and behavioral issues for a developing fetus, newborn, and child. In fact, more than 75% of newborns exposed to illicit drugs have major medical problems [2]. For example, neonatal abstinence syndrome, respiratory distress, low birth weight, and sudden infant death syndrome are all toxidromes associated with prenatal drug exposure. Furthermore, a mother with an illicit drug addiction may not be able to provide a safe and nurturing environment for the infant [3, 4]. Older children exposed to illicit drugs in utero also exhibit poor social adjustment, learning disabilities, and attention-focusing issues [4]. Therefore, the detection of illicit drug exposure in utero is crucial

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to identify and treat developmental difficulties, both medical and social, which the drug-exposed newborn and child may encounter.

Meconium is the first fecal matter passed by a newborn. It is a heterogeneous material composed of amniotic fluid, bile, lipids, and other waste materials that accumulate in the developing fetus starting around 12 weeks of gestation and continues to form until birth [5]. Thus, meconium analysis can yield information detailing a nearly 6-month window of possible drug exposure; meconium is also easily and noninvasively collected from soiled diapers. However, meconium analysis presents unique challenges; these are mostly associated with the matrix's lack of heterogeneity and its semisolid composition. The sticky, tar-like consistency of meconium necessitates the individual weighing of samples for analysis. Additionally, meconium requires large volumes of solvent and long extraction times with high-energy procedures in order to ensure the sample is completely extracted in contrast to a superficial extraction of the surface layer of the sample.

Meconium samples may be initially analyzed with an immunoassay kit-based technique to quickly screen a large number of samples; however, these kits often have high false-positive rates from similar molecules binding the antibodies. Additionally, the variation of meconium color can interfere with the immunoassay measurement also causing false-positives in intensely colored specimens. Therefore, samples that screen positive by immunoassay need to be confirmed via more specific methods, typically gas chromatography-mass spectrometry (GC-MS)- or liquid chromatography-mass spectrometry (LC-MS/MS)-based techniques. Yet another challenge of monitoring drugs of abuse in meconium is whether the confirmation methods target the correct metabolites as the metabolism of a fetus may create different metabolites than what are observed in adult urine. For example, *m*-hydroxybenzoylecgonine and *p*-hydroxymethamphetamine appear to be important metabolites of cocaine and methamphetamine in meconium, while they are not typically monitored in urine [6, 7].

Several extraction techniques have been reported in the literature for meconium drug testing by mass spectrometry-based methods. For example, ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), accelerated solvent extraction (ASE), and solid-phase extraction (SPE) have been utilized alone or in combination for meconium extraction prior to GC-MS or LC-MS/MS confirmation analysis [6, 8, 9]. The majority of extraction methods combine a method of heating or disrupting the meconium in a solvent to extract the analytes, followed by SPE to specifically isolate the analytes of interest. UAE and SPE methods were evaluated at MedTox Laboratories; however, depending on the solvent utilized for UAE, the extract was dark in color and not very clean (methanol), or only the surface of the meconium appeared to be extracted (acetonitrile). While SPE

cleaned the meconium samples significantly better than UAE alone, it is relatively expensive and time-consuming. Therefore, a method utilizing salting-out assisted liquid-liquid extraction (SALLE) aided by ceramic homogenizer shearing of the meconium was developed and is presented here.

Several salts can be utilized for SALLE to separate water miscible organic solvents from aqueous samples to directly analyze the extract by LC-MS/MS without the need for evaporation nor reconstitution [10]. To simultaneously extract cocaine, amphetamines, and their respective metabolites, ammonium acetate and acetonitrile were utilized as SALLE reagents. Cocaine (COC) and cocaethylene (CE) were quantified over a range of 1–500 ng/g, while benzoylecgonine (BE), *m*-hydroxybenzoylecgonine (*m*-OHBE), amphetamine (AMP), methamphetamine (MAMP), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA) were quantified over respective ranges of 5–1000 ng/g. Extraction recoveries ranged from 19% for the most polar analyte (*m*-hydroxybenzoylecgonine) to 102% for cocaine.

2 Materials

2.1 Solutions and Standards

1. 50:50 methanol/water (v/v): Add 100 mL of methanol and 100 mL of water to a glass container, cap tightly, and mix. Store at room temperature for up to 1 year.
2. 1 μg/mL COC-d_3 and CE-d_3, 3 μg/mL AMP-d_8, MAMP-d_{14}, MDA-d_5, and MDMA-d_5 internal standard spiking solution: Combine 100 μL of 1 mg/mL COC-d_3 stock and 1 mL of 0.1 mg/mL CE-d_3 stock, plus 300 μL of each 1 mg/mL stock of AMP-d_8, MAMP-d_{14}, MDA-d_5, and MDMA-d_5 in a 100 mL volumetric flask, and bring to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.
3. 30 μg/mL standard substock and control substock.
   (a) Standard substock: Combine 0.3 mL of each 1 mg/mL stock of AMP, MAMP, MDA, MDMA, BE, and *m*-OHBE in a 10 mL volumetric flask. Fill to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.
   (b) Control substock: Repeat **step 3a** with independent preparations of the standard solution stocks.
4. 25 μg/mL AMP, MAMP, MDA, MDMA, BE, and *m*-OHBE; 12.5 μg/mL COC and CE spiking standard and spiking control.

(a) Spiking standard: Combine 250 µL each of the 1 mg/mL AMP, MAMP, MDA, MDMA, BE, and *m*-OHBE stock standards, plus 125 µL of the 1 mg/mL COC and CE stock standards in a 10 mL volumetric flask. Fill to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.

(b) Spiking control: Repeat **Step 4a** with stock standards from an independent preparation of the standard solution stocks.

5. 10 µg/mL AMP, MAMP, MDA, MDMA, BE, and *m*-OHBE; 2.0 µg/mL COC and CE spiking standard: Combine 2 mL of the 30 µg/mL standard substock and 1.6 mL of the 25 µg/mL/12.5 µg/mL spiking standard in a 10 mL volumetric flask. Fill to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.
6. 2.5 µg/mL AMP, MAMP, MDA, MDMA, BE, and *m*-OHBE; 0.5 µg/mL COC and CE spiking control: Add 0.5 mL of the 30 µg/mL control substock and 0.4 mL of the 25 µg/mL/12.5 µg/mL spiking control to a 10 mL volumetric flask. Fill to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.
7. 0.25 µg/mL AMP, MAMP, MDA, MDMA, BE, and *m*-OHBE; 0.05 µg/mL COC and CE spiking standard and spiking control.
   (a) Spiking standard: Add 0.25 mL of the 10 µg/mL/2.0 µg/mL spiking standard to a 10 mL volumetric flask. Fill to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.
   (b) Spiking control: Add 1 mL of the 2.5 µg/mL/0.5 µg/mL spiking control to a 10 mL volumetric flask. Fill to volume with 50:50 methanol/water. Stable up to 1 year stored at 4 °C in glass screw-cap tubes with Teflon septa.
8. 3M ammonium acetate: Add approximately 150 mL of deionized water to a 500 mL volumetric flask. Weigh 115.6 g of ammonium acetate, and add to the volumetric flask. Dilute to volume with water, and mix. Store in amber bottle at room temperature for up to 3 months.
9. Mobile phase A, 0.1% formic acid: Add approximately 500 mL of deionized water to a 1 L volumetric flask. Add 1.0 mL of formic acid to the volumetric flask, dilute to volume with water, and mix. Stable when stored in a glass bottle at room temperature for up to 6 months.
10. Mobile phase B: Methanol, HPLC grade.

11. Autosampler wash #1, 0.1% formic acid in methanol: Using a graduated cylinder, add 1 L of methanol to a 1 L bottle. Add 1.0 mL of formic acid to the bottle, cover, and mix.
12. Autosampler wash #2, 90% water/10% methanol with 0.1% formic acid: Using a graduated cylinder, add 900 mL of deionized water to a 1 L glass bottle. Add 100 mL of methanol and 1 mL of formic acid to the bottle, cover, and mix.

2.2 Supplies and Equipment

1. Negative meconium (purchased or screened in-house from donor specimens) for the preparation of calibrators and controls.
2. Wooden applicators.
3. Ceramic homogenizers (Agilent or similar).
4. 15 mL polypropylene tubes, capped.
5. Multi-tube vortex (VWR VX-2500 or similar).
6. Centrifuge (Beckman Coulter Allegra 25R, or similar).
7. LC-MS vials and caps.
8. Waters HSS T3, 50 × 2.1 mm, 1.8 μm column.
9. Thermo BetaBasic C18 guard column, 10 × 2.1 mm, 5 μm.
10. LC-MS/MS system (Waters classic UPLC and Sciex 5500 triple quadrupole).

3 Methods

3.1 Calibrator and Control Preparation

1. Weigh 0.5 g of negative meconium into a labeled 15 mL tube for each calibrator and control (*see* **Note 1**).
2. Spike the tube labeled Standard 1 with 10 μL of the 0.25/0.05 μg/mL spiking standard (*see* **Note 2**). Final concentration is 5 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 1 ng/g COC, CE.
3. Spike the tube labeled Standard 2 with 20 μL of the 0.25/0.05 μg/mL spiking standard. Final concentration is 10 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 2 ng/g COC, CE.
4. Spike the tube labeled Standard 3 with 10 μL of the 10/2 μg/mL spiking standard. Final concentration is 200 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 40 ng/g COC, CE.
5. Spike the tube labeled Standard 4 with 20 μL of the 10/2 μg/mL spiking standard. Final concentration is 400 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 80 ng/g COC, CE.

6. Spike the tube labeled Standard 5 with 20 μL of the 25/12.5 μg/mL spiking standard. Final concentration is 1000 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 500 ng/g COC, CE.
7. Spike the tube labeled Low QC with 30 μL of the 0.25/0.05 μg/mL spiking control (*see* **Note 3**). Final concentration is 15 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 3 ng/g COC, CE.
8. Spike the tube labeled Medium QC with 20 μL of the 2.5/0.5 μg/mL spiking control. Final concentration is 100 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 20 ng/g COC, CE.
9. Spike the tube labeled High QC with 16 μL of the 25/12.5 μg/mL spiking control. Final concentration is 800 ng/g AMP, MAMP, BDA, MDMA, BE, *m*-OHBE, and 400 ng/g COC, CE.
10. Reserve one tube of meconium labeled as Standard 0 to contain no added analytes; process this sample in an identical manner to all calibrators and samples.
11. Reserve one tube of meconium labeled as Blank to contain no added analytes; do not add internal standard to this sample.

3.2 Extraction

1. Weigh 0.5 g of each meconium sample into a labeled 15 mL tubes (*see* **Note 4**).
2. Add 20 μL of the internal standard spiking solution into each calibrator, control, and sample tube.
3. Add 1.5 mL of 3M ammonium acetate into each tube (*see* **Note 5**).
4. Add 2 ceramic homogenizers to each tube (*see* **Note 6**).
5. Cap each tube, and vortex mix samples utilizing multi-tube vortex for 10 min at medium speed or until meconium samples appear to be liquefied.
6. Add 1.25 mL of acetonitrile to each tube, and recap (*see* **Note 7**).
7. Vortex mix for 15 min to extract (*see* **Note 8**).
8. Centrifuge tubes for 10 minutes at 1100 × *g*.
9. Transfer 200 μL of the supernatant to labeled LC-MS vials (*see* **Note 9**).
10. Dilute each vial with 400 μL of 0.1% formic acid.
11. Cap each vial and vortex mix prior to LC-MS/MS analysis.

3.3 LC-MS/MS Analysis

1. Autosampler temperature: 10 °C.
2. Injection volume: 8 μL.
3. Column temperature: 50 °C.

Table 1
Chromatographic gradient parameters

Time (min)	0.1% Formic acid in water (A), %	Methanol (B), %	Flow (mL/min)
0.00	70.0	30.0	0.500
0.25	70.0	30.0	0.500
0.70	63.0	37.0	0.500
1.00	50.0	50.0	0.500
1.40	50.0	50.0	0.500
1.50	10.0	90.0	0.500
1.70	70.0	30.0	0.500
2.00	70.0	30.0	0.500

4. Gradient is shown in Table 1 (*see* **Note 10**).
5. Ion source parameters: Gas temp = 550 °C, curtain gas = 35 psi, ion spray voltage = 5000 V, ion source gases = 55, declustering potential = 90 V, entrance potential = 10 V, and exit potential = 12 V.
6. Mass spectrometer parameters: Compound-dependent parameters are listed in Table 2 (*see* **Note 11**).
7. Figure 1 presents a representative chromatogram of a standard containing all analytes and internal standards.

4 Notes

1. The meconium may stick to walls of the tube; therefore, it may be necessary to centrifuge tubes briefly to get the meconium to the bottom of tube where it is available for solvent extraction.
2. The SALLE may not separate the acetonitrile as efficiently if too much methanol is added by utilizing larger spiking volumes of the calibrator stock solutions.
3. Due to the difficulty in preparing a homogeneous mixture of meconium, calibrators and controls must be prepared individually by first weighing out the appropriate amount of meconium (0.5 g for this assay) and spiking with standards to the desired concentration. Low, mid, and high controls are typically prepared at approximately 3× the lowest calibrator for the low and at approximately 80% of the highest calibrator for the high control. The mid control generally approximates the geometric mean or logarithmic mean of the dynamic range of the assay.

Table 2
Monitored transitions

Analyte	Precursor ion (*m/z*)	Quantitative product ion		Qualitative product ion		Retention time (min)
		m/z	Collision energy (V)	*m/z*	Collision energy (V)	
AMP	136.1	119.1	17	91.1	47	0.60
MAMP	150.1	119.1	15	91.2	40	0.60
MAMP_isotopologue[a]	152.1	121.1	15	93.2	40	0.60
MDA	180.1	105.1	33	133.2	25	0.60
MDMA	194.5	163.1	22	133.1	25	0.60
COC	304.5	182.1	41	150.1	35	1.00
BE	290.1	168.1	28	105.1	40	0.90
m-OHBE[b]	306.1	168.1	27	121.1	38	0.60
CE	318.5	196.2	41	150.1	32	1.30
AMP-D_8 (IS)	144.1	127.1	12	–	–	–
MAMP-D_{14} (IS)	164.1	98.1	35	–	–	–
MDA-D_5 (IS)	185.1	138.1	25	–	–	–
MDMA-D_5 (IS)	199.1	165.1	18	–	–	–
COC-D_3 (IS)	307.1	185.1	25	–	–	–
BE-D_8 (IS)	298.1	171.1	27	–	–	–
CE-D_8 (IS)	326.4	204.2	28	–	–	–

[a] *See* **Note 11**
[b] AMP-d_8 internal standard is utilized for *m*-OHBE quantitation

4. Meconium is a viscous heterogeneous substance; therefore, it is important to mix well prior to removing a sample aliquot. This can be done by kneading the meconium with the wooden applicator.
5. The 3M ammonium acetate is utilized to solvate the meconium, and the high salt concentration allows acetonitrile to be utilized for liquid-liquid extraction. Ammonium acetate is also a LC-MS compatible buffer in case of aqueous phase contamination into the organic extract.
6. The ceramic homogenizers are utilized to break up and liquefy the meconium prior to liquid extraction. Ceramic beads (2.8 mm, e.g., ChromTech) have also successfully been utilized for extraction of smaller meconium sample sizes (0.1 g) by our laboratory. The use of ceramic homogenizers increased

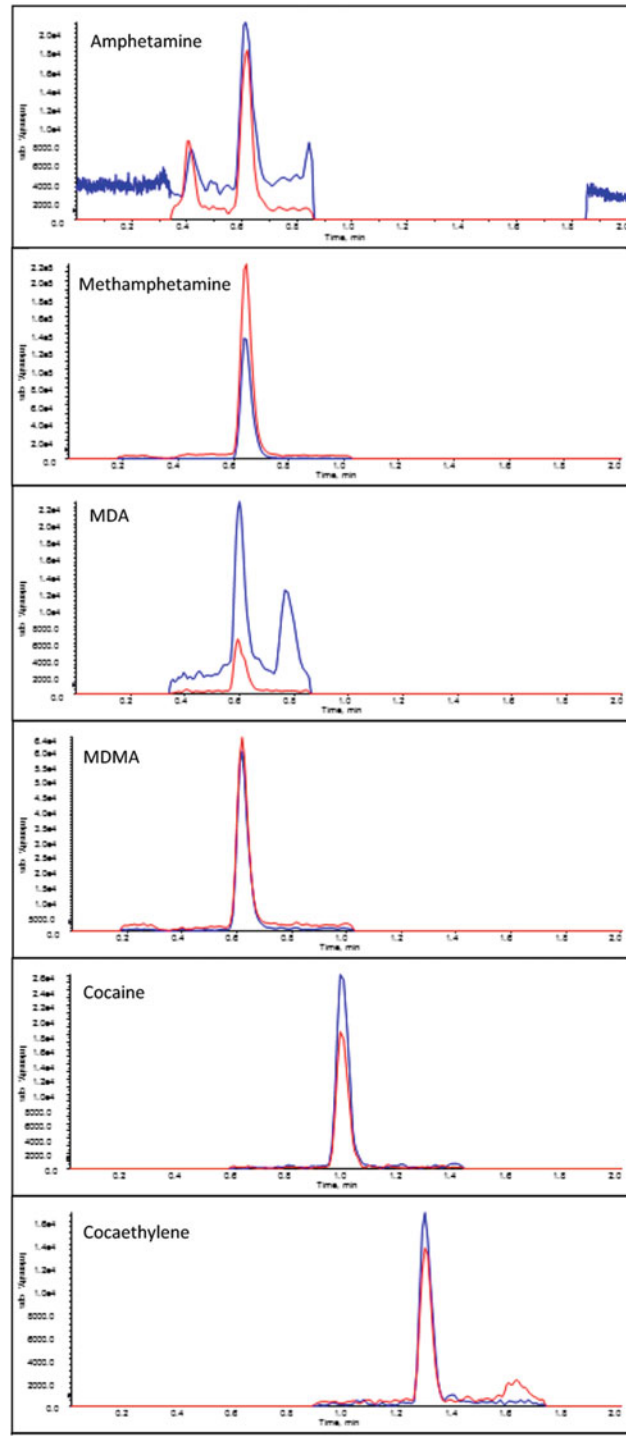

Fig. 1 A representative chromatogram of a standard containing all analytes and internal standards

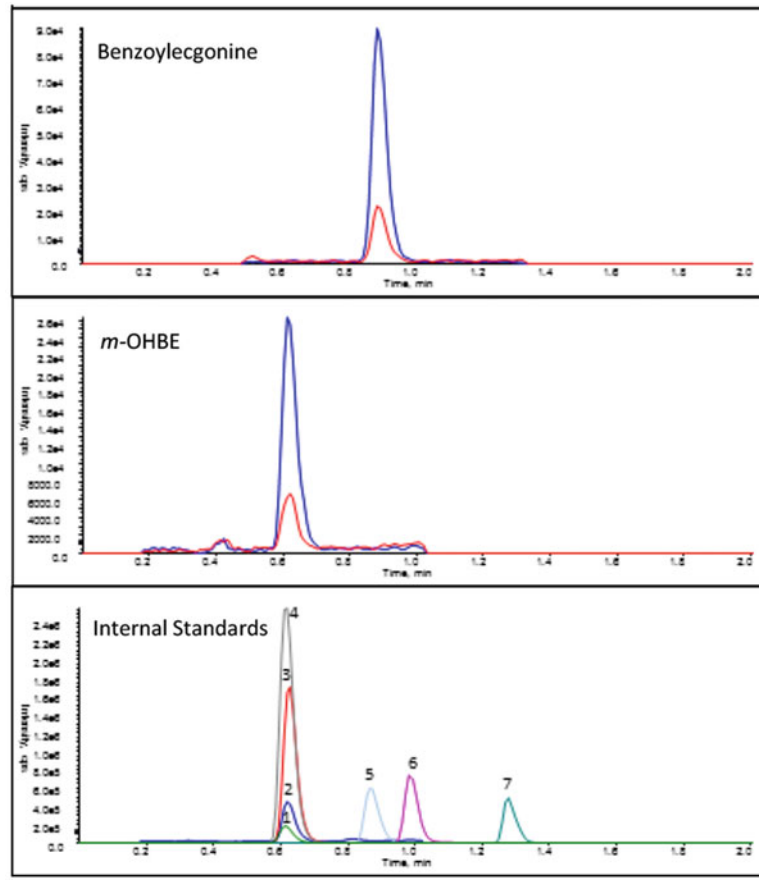

Fig. 1 (continued)

extraction efficiency of drugs from authentic meconium samples by an average of ~43% compared to sonication/mixing with organic solvent alone.

7. For more polar analytes, a small amount of methanol can be added to the acetonitrile to improve recoveries from the aqueous layer. However, if the methanol concentration is greater than ~5%, the aqueous and organic layers will not separate.
8. Following vortex mixing, two layers should form in the tube. The bottom layer will consist of the aqueous buffer, which should contain most of the dark green color from the meconium. The upper layer will be the acetonitrile containing analytes and will likely have a yellow color of varying intensities based on the meconium specimen.
9. Following centrifugation, three layers may be visible, the same as in **Note 8**, and an additional layer of meconium debris at the bottom of the tube.
10. Injection volume may vary based on instrument sensitivity.

11. Methamphetamine levels in excess of 10× the upper limit of quantitation (1000 ng/g) have frequently been observed by our laboratory in clinical specimens, at which point the quantitative accuracy, peak shape, retention time, and transition ratios begin to fail. Samples can be re-extracted with dilution to fall within the linear range; however, meconium samples are often limited, and results are time-sensitive as a newborn will likely only be in the hospital for a couple days. An isotopologue method for the quantitation of methamphetamine and amphetamine in urine has recently been validated [11], in which additional transitions are monitored simultaneously for methamphetamine and transitions shifted 2 amu to monitor the isotopologues containing two carbon-13 atoms. The relative abundance of the isotopologues is much lower than the base compound, thereby decreasing the sensitivity for high concentration samples. The same isotopologue methodology applied for meconium methamphetamine has increased the linear range 100-fold.

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Chapter 20

Detection of In Utero Cannabis Exposure in Umbilical Cord Tissue by a Sensitive Liquid Chromatography-Tandem Mass Spectrometry Method

Fang Wu, Triniti L. Jensen, and Gwendolyn A. McMillin

Abstract

In utero exposure to cannabis may cause various short- and long-term health problems in newborns, such as low birth weight and neonatal withdrawal syndrome. Drug testing with umbilical cord tissue can be used to identify in utero exposure to cannabis. Here, we described a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that simultaneously quantifies four cannabinoids in umbilical cord tissue, including tetrahydrocannabinol (THC), 11-nor-Δ9-carboxy-THC (THC-COOH), cannabinol (CBN), and 11-hydroxy-THC (11-OH-THC). Umbilical cord specimens are weighed and homogenized, and cannabinoids are extracted using anion exchange solid-phase extraction columns (AX-SPE). Liquid chromatography separation is performed, and quantitative results are obtained using LC-MS/MS.

Key words Cannabis exposure, THC, THC-COOH, 11-OH-THC, CBN, Umbilical cord, LC-MS/MS

1 Introduction

Cannabis is the most commonly used illicit drug in the United States [1]. According to the results of a 2015 survey, its use is widespread among young Americans. Among pregnant women, 4.7% admitted to using cannabis in the past month [2]. Adverse effects of in utero exposure to cannabis on growth, length of gestation, intelligence, and cognitive functions have been reported by a large number of studies [3–8]. Moreover, impulsivity and impaired executive function have been noted in children exposed to cannabis during gestation [1, 9]. Drug testing has become a useful tool for timely detection of in utero exposure to cannabis, and testing results can be used to guide social management and treatment.

Urine [1], hair [10], meconium [11, 12], and umbilical cord [13–15] have been used for detection of drug exposure in

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newborns. Urine results typically reflect drug exposure in the preceding 2–3 days. Neonatal hair can detect cumulative exposure to various drugs during the third trimester [16]. However, hair testing is not always feasible for newborns due to limited hair volume. Meconium, the first stool from newborns, is able to detect in utero drug exposure up to 20 weeks after maternal use. It has become a well-established specimen to assess in utero exposure to drugs including cannabinoids [12, 17, 18]. However, meconium is not available in some cases (10–20%) [11] because of early passing prior to birth; this is particularly seen in the setting of fetal stress caused by maternal drug use during gestation. Therefore, alternative specimen types are necessary to determine in utero exposure to cannabis as well as other licit or illicit drugs. Umbilical cord tissue is a relatively new specimen type, serving as an alternative to meconium for in utero drug exposure testing. Advantages of umbilical cord tissue include the amount available for testing [14] as well as easy specimen collection for all newborns at birth. If prior suspicion of drug use is indicated, drug testing can be performed immediately after birth with umbilical cord tissue, making results available faster than meconium testing results, in most cases. However, the timing and drug deposition patterns in umbilical cord tissue are not well studied [19, 20].

Available literature currently provides little published data on testing for cannabinoids in umbilical cord tissue [15]. This chapter outlines an LC-MS/MS method for identification and quantification of four cannabinoids, including tetrahydrocannabinol (THC), 11-nor-Δ9-carboxy-THC (THC-COOH), cannabinol (CBN), and 11-hydroxy-THC (11-OH-THC), in umbilical cord tissue. Since metabolic patterns of cannabinoids in umbilical cord tissue are not well known, results from this assay could be useful to detect and characterize in utero cannabis exposure. In this chapter, the authors describe umbilical cord tissue homogenization, solid-phase extraction (SPE), liquid chromatographic (LC) separation, as well as mass spectrometric (MS/MS) detection with the focus on extraction procedure since extraction of cannabinoids from umbilical cord tissue is a bottleneck in the procedure. The extraction method described in this chapter could recover 60–86% of the four cannabinoids from umbilical cord tissue.

2 Materials

Label all reagents, calibrators, and controls with contents, date prepared, and expiration date. All organic solvents are HPLC grade or greater. Water should be clinical laboratory reagent grade or higher.

2.1 Instrumentation and Supplies (See Note 1)

1. Class A grade glassware.
2. Pipettes.
3. Mini food chopper (e.g., Joint Legend Group, model# JE3049).
4. Stainless steel beads (5.6 mm or similar size).
5. Tissue homogenizer (e.g., Bead Ruptor).
6. Eppendorf microcentrifuge.
7. VWR Advanced Multi-tube Vortexer.
8. Biotage Evolute® AX-SPE columns (60 mg/3 mL).
9. Positive pressure manifold.
10. LC vials (maximum recovery).
11. Nitrogen evaporator/concentrator.
12. Phenomenex Kinetex Evo C18 column (2.1 × 50 mm, 2.6 μm particle size).
13. CTC PAL HTC-xt-DLW autosampler.
14. Agilent 1260 infinity series binary pump, degasser, and column oven.
15. AB SCIEX Triple Quad™ 5500 mass spectrometer.
16. AB SCIEX Analyst and MultiQuant software.

2.2 Stock Non-deuterated Standard Solutions

Four liquid standards containing 1 mg/mL THC, THC-COOH, 11-OH-THC, and CBN, respectively, are used to prepare stock 1. A tenfold serial dilution is made from stock 1 to obtain stocks 2 and 3. Aliquot and store all stock solutions frozen (−65 to −75 °C).

1. Stock 1: 1 μg/mL THC, THC-COOH, 11-OH-THC, and CBN in methanol. Add 5 mL of methanol to a 10 mL glass volumetric flask. Add 10 μL of each 1 mg/mL liquid standard into the flask. Fill flask with methanol to the 10 mL line, and mix well.
2. Stock 2: 100 ng/mL THC, THC-COOH, 11-OH-THC, and CBN in methanol. Add 1 mL of stock 1 to a 10 mL glass volumetric flask. Fill flask with methanol to the 10 mL line, and mix well.
3. Stock 3: 10 ng/mL THC, THC-COOH, 11-OH-THC, and CBN in methanol. Add 1 mL of stock 2 to a 10 mL glass volumetric flask. Fill flask with methanol to the 10 mL line, and mix well.

2.3 Stock Non-deuterated Control Solutions (See Note 2)

Liquid standards containing 1 mg/mL THC, THC-COOH, 11-OH-THC, and CBN and 100 μg/mL THC-COOH glucuronide, respectively, are used to prepare stock 4. A tenfold serial dilution is made from stock 4 to obtain stocks 5 and 6. Aliquot and store all stock solutions in freezer (−65 to −75 °C).

1. Stock 4: 1 μg/mL THC, 11-OH-THC, and CBN and 1.51 μg/mL THC-COOH-glucuronide in methanol. Add 5 mL of methanol to a 10 mL glass volumetric flask. Add 151 μL of THC-COOH glucuronide and 10 μL of THC, 11-OH-THC, and CBN 1 mg/mL standard into the flask. Fill flask with methanol to the 10 mL line, and mix well.
2. Stock 5: 100 ng/mL THC, 11-OH-THC, and CBN and 151 ng/mL THC-COOH-glucuronide in methanol. Add 1 mL of stock 4 to a 10 mL glass volumetric flask. Fill flask with methanol to the 10 mL line and mix well.
3. Stock 6: 10 ng/mL THC, 11-OH-THC, and CBN and 15.1 ng/mL THC-COOH-glucuronide in methanol. Add 1 mL of stock 5 to a 10 mL glass volumetric flask. Fill flask with methanol to the 10 mL line and mix well.

2.4 Stock Deuterated Internal Standard Solution

1. Stock 7: 100 ng/mL THC-d3, THC-COOH-d3, 11-OH-THC-d3, and CBN-d3 in methanol. Add 5 mL of methanol to a 10 mL glass volumetric flask. Add 10 μL of each 100 μg/mL standard of THC-d3, THC-COOH-d3, 11-OH-THC-d3, and CBN-d3, respectively, to the flask. Fill the flask with methanol to the 10 mL line, and mix well before use. Aliquot into 2 mL glass vials and store all stock solutions in freezer (−65 to −75 °C).

2.5 Buffers and Mobile Phases

1. SPE column wash buffer 1: 1% ammonium hydroxide in water. Add 80 mL of water to a 100 mL graduated cylinder. Add 1 mL of ammonium hydroxide into the graduated cylinder. Fill to 100 mL with water. Mix before use.
2. SPE column wash buffer 2: methanol.
3. SPE column elution buffer: 2% acetic acid in methanol. Add 80 mL of methanol to a 100 mL graduated cylinder. Add 2 mL of glacial acetic acid into the graduated cylinder. Fill to 100 mL with methanol. Mix before use. SPE column elution buffer should be prepared fresh daily and stored at room temperature.
4. Reconstitution buffer: 60:40 methanol/water. Add 60 mL of methanol to a 100 mL graduated cylinder. Fill to 100 mL with water. Mix before use.
5. Hydrolysis buffer: 0.5 M NaOH. Add 100 mL of water to a 1 L graduated cylinder. Weigh 20 g of NaOH and transfer to the cylinder. Fill to 1 L with water. Mix well before use.
6. HPLC mobile phase A: 5 mM ammonium bicarbonate in water, pH 9.5. Add 100 mL of water to a 1 L graduated cylinder. Weigh 0.40 g of ammonium bicarbonate and transfer to the cylinder. Add water to up to the 900 mL mark. Mix and adjust pH to 9.5 with ammonium hydroxide. Fill to 1 L with

water. Mobile phase A is stable at room temperature for at least 10 days.

7. HPLC mobile phase B: methanol. Mobile phase B is stable at room temperature for at least 14 days.

2.6 Drug-Free Umbilical Cord Pool

1. Evaluate residual umbilical cord tissue samples using the presented LC-MS/MS method to ensure absence of detectable cannabinoids. De-identify residual umbilical cord tissue samples. Pool approximately 80 residual blank umbilical cord tissue samples. Homogenize drug-free umbilical cord pool using a mini food chopper (approximately 1/2 cm cubes). Fill individual 50 mL polypropylene conical centrifuge tubes (~30 g per tube). Sixty grams of blank umbilical cord tissue is able to accommodate approximately 1 week of testing if one run is performed per day. Store in freezer (−65 to −75 °C); blank umbilical cord tissue is stable at these temperatures for at least 1 year and up to two freeze-thaw cycles.

3 Methods

Figure 1 illustrates the schematic workflow of specimen handling.

3.1 Sample, Calibrator, and Control Weigh Out

1. Cut umbilical cord tissue using a clean razor blade for each sample. Discard used razor blades in the sharp container. Razors should never be reused.
2. Weigh 1.0 ± 0.1 g of each patient umbilical cord tissue (*see* **Note 3**) into individual 5 mL conical polypropylene tubes with screw top caps.

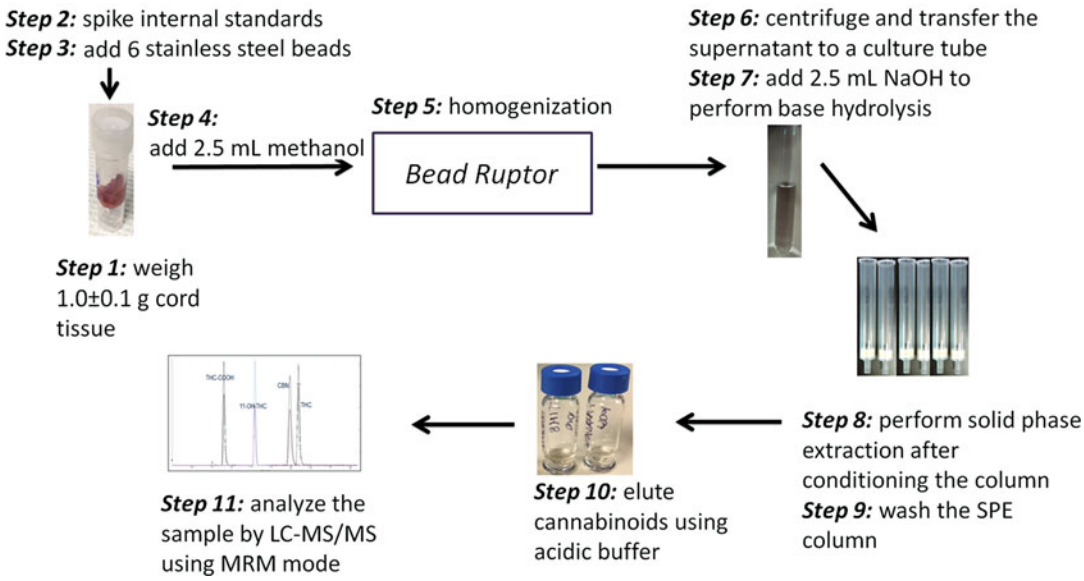

Fig. 1 Schematic workflow for processing and analyzing umbilical cord tissue

3. Label eight 5 mL conical polypropylene tubes with screw top caps from 1 to 8. Tubes 1–5 for calibrator preparation and tubes 6–8 for controls.
4. Weigh 1.0 ± 0.1 g of pooled blank cord into tubes 1–8 (*see* **Note 4**).
5. Spike internal standard solution: Add 25 μL of stock deuterated internal standard solution (stock 7) to tubes 1–8 and patient samples.

3.2 Calibrator and Control Spiking

A calibration curve is generated with the following calibrator concentrations: 0.2, 0.5, 1, 3, and 5 ng/g in drug-free umbilical cord tissue. Three controls are included at 0, 0.25, and 4 ng/g (i.e., negative, low, and high) (*see* **Note 5**).

1. Spike tube 1 with 20 μL of stock 3 to create calibrator 1 (0.2 ng/g).
2. Spike tube 2 with 50 μL of stock 3 to create calibrator 2 (0.5 ng/g).
3. Spike tube 3 with 10 μL of stock 2 to create calibrator 3 (1 ng/g).
4. Spike tube 4 with 30 μL of stock 2 to create calibrator 4 (3 ng/g).
5. Spike tube 5 with 50 μL of stock 2 to create calibrator 5 (5 ng/g).
6. Spike tube 6 with 25 μL of stock 6 to create a low QC (0.25 ng/g).
7. Spike tube 7 with 40 μL of stock 5 to create a high QC (4 ng/g).
8. Spike tube 8 with stock 7 (internal standard) *only*. Tube 8 without non-deuterated standard solution is used as a negative QC.

3.3 Homogenization and Base Hydrolysis

1. Add six stainless steel beads into each tube.
2. Add 2.5 mL of methanol into each tube.
3. Store samples in freezer (−65 to −75 °C) for 10 min.
4. Remove samples from freezer and immediately place them in a homogenizer.
5. Homogenize samples for 4 min at 5 m/s in 30 s intervals with 30 s rest periods to minimize heat exposure to the tissue.
6. Centrifuge homogenates at 20,598 × *g* (14,000 rpm) and 4 °C for 10 min in a microcentrifuge.
7. Transfer supernatants to individual pre-labeled 10 mL culture tubes.
8. Add 2.5 mL of 0.5 M NaOH into each supernatant.

9. Incubate samples at ambient temperature (23–25 °C) on a multi-tube vortexer at a shaking frequency of 700 rpm for 45 min while shaking to convert THC-COOH glucuronide to free THC-COOH.

3.4 SPE Extraction

1. Place SPE columns on a positive pressure manifold.
2. Condition individual SPE columns with 1 mL of methanol followed by 1 mL of water.
3. Load supernatants onto SPE columns (*see* **Note 6**).
4. Wash individual SPE columns with 1 mL of 1% ammonium hydroxide wash buffer.
5. Wash individual SPE columns with 1 mL of methanol.
6. Dry SPE columns at 5 kPa for 1 min to remove residual methanol.
7. Elute by gravity into individual LC vials with two 0.6 mL fractions of 2% acetic acid SPE elution buffer.
8. Dry the eluent at 40 °C under a gentle nitrogen stream with a CEREX concentrator 48 or similar evaporator for 20 min (*see* **Note** 7).
9. Reconstitute each sample in 200 µL of reconstitution buffer.
10. Vortex the vials for 5 min at 1200 rpm before LC-MS/MS analysis.

3.5 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis (See Note 8)

LC-MS/MS analysis is described for an AB SCIEX Triple Quad™ 5500 mass spectrometer interfaced with CTC PAL HTC-xt-DLW autosampler and Agilent 1260 Infinity Series binary pump, degasser, and column oven.

1. Column: Kinetex Evo C18 column (2.1 × 50 mm, 2.6 µm particle size).
2. LC gradient is described in Table 1. Divert the LC eluent to waste for the first 0.8 min and the final 0.5 min.
3. LC flow rate: of 0.5 mL/min.
4. Injection volume: 40 µL.
5. MS/MS mode: negative electrospray ionization mode.
6. MS/MS parameters are shown in Table 2 (*see* **Note 9**).
7. Collect data using scheduled multiple reaction monitoring (MRM) mode. Use AB SCIEX Analyst software or comparable for instrument control. Set two transition ions to monitor each drug analyte, as shown in Table 3.
8. Create a worklist of specimens to be tested.
9. Equilibrate the LC column at the starting conditions (40:60 water/methanol) for 5–10 min before initiating the run. Hold the column and autosampler at 28 °C and 4 °C, respectively.

Table 1
Liquid chromatography separation gradient[a]

Time (min)	%A	%B
0	40	60
0.5	40	60
1.2	20	80
2.3	20	80
2.7	10	90
3.2	10	90
3.21	5	95
4	5	95
4.01	40	60
4.7	40	60

[a]A, 5 mM ammonium bicarbonate in water, pH 9.5; B, methanol; flow rate used is 0.5 mL/min

Table 2
Mass spectrometry parameters

Mass spectrometry parameters	
Instrument mode	Negative
Curtain gas (psi)	30
Collision gas (psi)	10
Ion spray voltage	−4000
Temperature (°C)	550
Nebulizer gas (psi)	25
Heater gas (psi)	30

10. Verify the acquisition worklist for accuracy.
11. Apply the method to the batch. Figure 2 shows representative LC separation and extracted ion chromatograms of THC, THC-COOH, 11-OH-THC, and CBN from an authentic clinical sample.

3.6 Data Analysis

1. Construct calibration curves via linear least-squares regression with $1/x$ weighting factor.
2. Review calibrators (*see* **Note 10**). Review calibrators for any missing internal standards. Review retention times (RTs) for

Table 3
Drug testing components, multiple reaction monitoring transitions, retention time (RT), and internal standards[a]

Non-deuterated analytes/deuterated internal standards	RT (min)	MRM transitions for non-deuterated analytes	MRM transitions for deuterated analytes
THC/THC-d3	3.2	313.1 → 245.1 313.1 → 191.1	316.0 → 248.0 316.0 → 194.0
THC-COOH/THC-COOH-d3	1.4	343.1 → 245.0 343.1 → 191.0	346.0 → 248.2 346.0 → 194.0
11-OH-THC/11-OH-THC-d3	2.1	329.1 → 268.1 329.1 → 173.0	332.1 → 271.0 332.1 → 173.0
CBN/CBN-d3	3.0	309.3 → 222.1 309.3 → 171.0	312.0 → 282.0 312.0 → 171.0

[a]The LC-MS/MS cutoff used is 0.2 ng/g for each compound

analytes and internal standards for any flags, and review integration and chromatogram quality.

3. Review QCs. QC values should be within ±20% of the target concentration (*see* **Note 11**).
4. Review patient samples (see **Note 12**).

4 Notes

1. The presented items are in the order of sample preparation, LC, and MS. Similar instruments can be used.
2. Stock non-deuterated control solution should be prepared with a different lot number of standard solutions than those used for stock standard preparation. Quality control samples contain glucuronidated THC-COOH. Under basic conditions, THC-COOH glucuronide is converted to free drug, THC-COOH. Hydrolysis efficiency is monitored by conversion yield. Higher concentration per weight of THC-COOH glucuronide is added to the stock to account for the weight difference compared to post hydrolysis-free THC-COOH (molecular weight is 66.2% of the glucuronidated drug).
3. Collect at least 6 in. of umbilical cord. For sample preparation, 1.0 ± 0.1 g of tissue is needed. Drain and discard any blood, and rinse the exterior of the cord segment with normal saline or water. Samples received soaking in fluid, as well as samples that have been excessively scoured or dried have an increased risk of false-negative results.
4. Use different lot numbers of blank cord to prepare calibrators and controls.

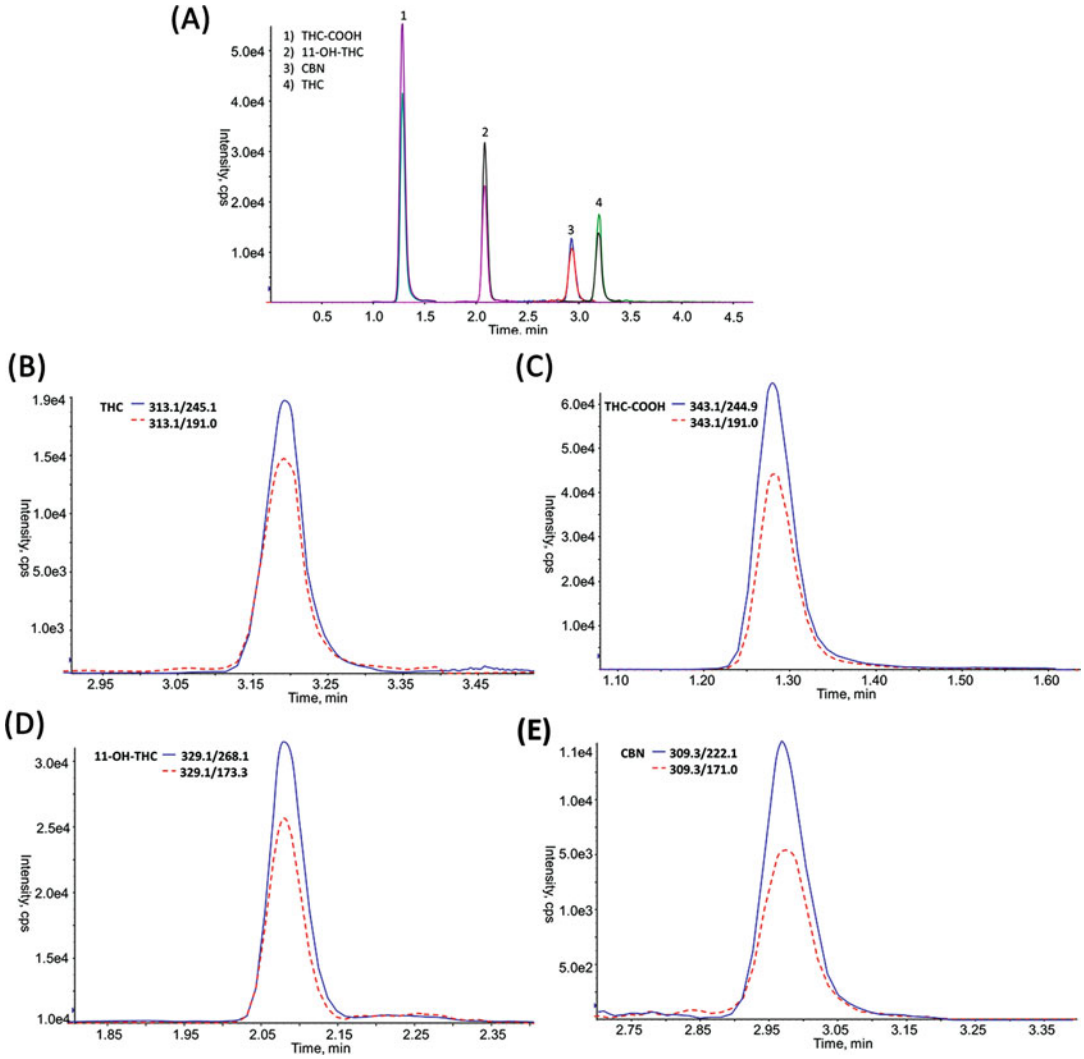

Fig. 2 Representative LC separation (**a**) and extracted ion chromatograms with ion transitions noted for (**b**) THC (1.3 ng/g), (**c**) THC-COOH (17.5 ng/g), (**d**) 11-OH-THC (0.9 ng/g), and (**e**) CBN (0.3 ng/g) from an authentic clinical sample

5. Calibrators and QCs should be performed in the same manner as the patient samples.
6. In order to achieve high recoveries, do not apply too much pressure to SPE columns and allow supernatants to pass through SPE columns at 1 drop/4 s.
7. Dry completely but do not overdry! If there is solvent remaining in the vials after 20 min, dry the sample under recommended conditions for a few more minutes (i.e., 2–5 min) to completely evaporate the solvent.
8. Complete the required instrument maintenance according to manufacturer's instruction prior to injecting the controls and

test specimens. Calibrators and controls must be injected prior to patient samples.

9. MS parameters were optimized via direct infusion of a mixture of standards.
10. The calibrators must have acceptable chromatography, retention time (RT), area counts, and ion ratios. Acceptable criteria for RT, ion ratios, and chromatography are listed below. If the ion ratios, RT, and chromatography are outside of the range, the specimen must be re-injected, re-extracted, or reported as "not detected." Manually integrate any compounds not found automatically.
    (a) RT must be ±10% of the target of RT for the specimen to be considered "present."
    (b) Ion ratios must be ±30% of expected values.
    (c) Ensure the correct peaks are identified in the chromatography and no other suspected peaks closer to the target RT for each compound.
11. Negative control must demonstrate the presence of all deuterated analytes, meet the acceptable criteria, and be negative for all other non-deuterated analytes (<LOD).
12. All target analytes must meet the acceptable criteria as described for calibrators.

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Chapter 21

Quantitation of Ethyl-β-D-Glucuronide in Human Umbilical Cord Tissue by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Simuli L. Wabuyele and Gwendolyn A. McMillin

Abstract

In utero exposure to alcohol may adversely affect the development of the embryo or fetus and result in adverse outcomes known as fetal alcohol spectrum disorders (FASD) which encompass a range of physical, behavioral, and cognitive impairments in the newborn. Since maternal self-reports are often unreliable, biomarkers of gestational alcohol consumption are necessary for accurate identification of exposed newborns at risk. Ethyl-β-D-glucuronide (EtG) is a minor phase II metabolite of ethyl alcohol (ethanol), formed by enzymatic conjugation of ethanol with glucuronic acid in the liver. As a direct biomarker for alcohol, detection of EtG in neonatal biological matrices provides accurate identification of maternal alcohol consumption and fetal alcohol exposure during pregnancy. This chapter describes the quantitation of EtG in human umbilical cord tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Key words Ethyl glucuronide (EtG), LC-MS/MS, Prenatal alcohol exposure, Umbilical cord, Newborn drug testing

1 Introduction

Alcohol is widely consumed in the United States, and it's the second most commonly abused legal psychoactive substance in pregnancy after nicotine [1]. According to the 2015 Nation Survey on Drug Use and Health estimates, among pregnant women aged 15–44, 9.3% reported alcohol consumption in the past month, 4.6% reported binge drinking (i.e., ≥4 drinks on the same occasion on at least 1 day in the past 30 days), and 0.8% reported heavy drinking (i.e., binge drinking on the same occasion on each of 5 or more days in the past 30 days) [2]. Alcohol consumption during pregnancy may severely affect the development of the embryo or fetus and result in adverse outcomes known as fetal alcohol spectrum disorders (FASD), which encompass a range of physical, neurobehavioral, and cognitive impairments in the exposed

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newborn [3]. FASD and the associated secondary disabilities may have lifelong implications which may result in substantial health care (medical), economic, and social costs [4]. Early identification of in utero exposure may aid in early diagnosis of FASD and is necessary for proper and timely follow-up of exposed newborn [5, 6]. Given that maternal-self reporting of alcohol use is often unreliable due to do underreporting, a reliable biomarker of alcohol abuse is necessary for accurate detection of in utero alcohol exposure [6].

Upon ingestion, most of the ethanol (about 90–98%) undergoes oxidative metabolism in the liver and <1% undergoes non-oxidative metabolism, whereas a small amount (2–5%) is excreted unchanged in urine, breath, and sweat (Fig. 1) [7]. Ethyl-β-D-glucuronide (EtG) is a minor phase II metabolite of ethyl alcohol (ethanol) formed by enzymatic conjugation of ethanol with glucuronic acid by UDP-glucuronosyltransferase in the liver [8, 9]; therefore, EtG is a direct biomarker for alcohol exposure [10]. Other direct ethanol biomarkers include ethyl sulfate (EtS), fatty acid ethyl esters (FAEE), and phosphatidylethanol (PEth) (Fig. 1) [10].

EtG has been determined to have high sensitivity and specificity for detection of acute and chronic ethanol exposure in maternal matrices [11, 12]; however, newborn specimens provide a direct

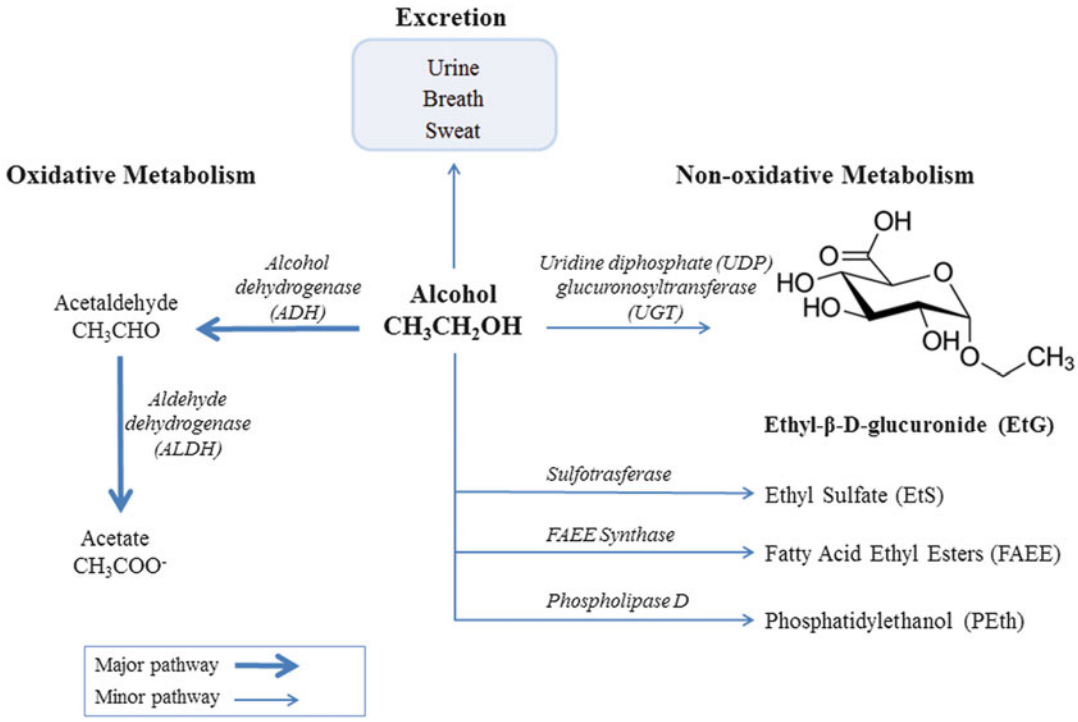

Fig. 1 Alcohol metabolism (oxidative and non-oxidative pathways) and excretion

measure of fetal alcohol exposure. EtG has been detected in meconium [13, 14], placenta and fetal tissue [15], and umbilical cord tissue [16] for identification of in utero alcohol exposure [17]. Umbilical cord specimen testing is a useful alternative to meconium due to several distinct advantages. Meconium can be unavailable for testing if it is passed in utero or passage is delayed and may require multiple collections over several days. However, umbilical cord is universally available in sufficient amounts immediately after birth, so that it can be easily and noninvasively collected in a single step for testing [16, 18, 19].

Currently, the number of analytical methods available for detection of EtG in umbilical cord specimens is limited. To our knowledge only one method has been reported in the peer-reviewed literature [16]. In this chapter, the quantitation of EtG in human umbilical cord tissue by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) for assessment of in utero alcohol exposure is described. Umbilical cord tissue specimens were sliced and weighed. Deuterated internal standard was added, and samples were homogenized and then centrifuged. The supernatants were subject to anion exchange solid-phase extraction (SPE) for cleanup prior to analysis and detection by LC-MS/MS.

2 Materials

Label all reagents, calibrators, and controls with contents, lot number, date prepared, expiration dates, and initials of preparer. Use LC grade or LC-MS grade reagents and follow guidelines/regulations for proper handling, storage, and disposal of hazardous waste. Note that similar equipment and supplies to those listed here can be used as available.

2.1 Supplies

1. Stir sticks, wooden, 5½″.
2. 50 mL conical sterile polypropylene centrifuge tubes with caps.
3. 7.0 mL polypropylene screw cap tubes with caps (Omni International™, Kennesaw, GA).
4. Large plastic antistatic weigh boats.
5. Vials, clear, silanized, Snap-it Cap, 12 × 32 mm, 1.5 mL.
6. Caps, Snap w/pre-slit, blue PTFE/white silicone.
7. Single edge razor blades.
8. 16 × 100 mm, 14 mL culture tubes, borosilicate glass.
9. Anion exchange solid-phase extraction (SPE) cartridges, 500-mg bed in a 6 mL reservoir (quaternary amine with chloride counter ion sorbent).

10. Synergi™ Hydro RP, C18 column, 50 mm × 3.0 mm, 2.5 μm.
11. Ultra HPLC In-Line Filter, 0.5 μm depth filter × 0.004″ ID.
12. 5.6 mm stainless steel UFO beads for Biotage® Bead Ruptor.
13. Mini food chopper/processor, 1.5-cup capacity.
14. Standard glassware, pipette tips, and other supplies.

2.2 Solutions

1. Elution solvent: 2% formic acid in methanol. Add 2 mL of 98% formic acid to 100 mL of methanol. Elution solvent must be freshly prepared prior to its use in each batch run.
2. Mobile phase A: 0.1% formic acid in water. Add 1 mL of 98% formic acid to 1 L of ultrapure water. Stable for 10 days at room temperature.
3. Mobile phase B: 0.1% formic acid in acetonitrile. Add 1 mL of 98% formic acid to 1 L of acetonitrile. Stable for 14 days at room temperature.
4. Autosampler wash solvent 1: 0.1% formic acid in 90:10 water/acetonitrile. Add 1 mL of 98% formic acid and 100 mL of acetonitrile to 900 mL of ultrapure water. Stable for 14 days at room temperature (*see* **Note 1**).
5. Autosampler wash solvent 2: 60:20:20 isopropanol/acetonitrile/methanol. Mix 600 mL of isopropanol (2-propanol), 200 mL acetonitrile, and 200 mL of methanol in a 1 L bottle. Stable for 14 days at room temperature (*see* **Note 1**).

2.3 Samples (see Note 2)

1. Patient specimen: Collect at least 6 in. of umbilical cord for testing. Excess cord blood should be drained and exterior of the cord segment rinsed with saline solution or water and patted dry.
2. Pooled drug-free (blank) umbilical cord: De-identify residual umbilical cord tissue specimens and screen for the presence of EtG using the LC-MS/MS method described here. Pool about 80–100 drug-free human umbilical cord samples. Using the mini food chopper/processor, cut the umbilical cords into approximately 0.5 cm cubes. Aliquot the pooled umbilical cord tissue into 50 mL polypropylene tubes (~30 g per tube). Sixty grams of blank cord is able to accommodate approximately 1 week of testing if one run is performed per day. Pooled drug-free (blank) umbilical cord is stable at freezer (−65 to −80 °C) temperatures for at least 1 year and up to two freeze-thaw cycles.

2.4 Standards and Calibrators

1. Standard stock solution: 50 μg/mL EtG in methanol. Add 500 μL of 1.0 mg/mL EtG to 5 mL of methanol in a 10 mL Class A volumetric flask. Fill to volume with methanol and mix well.

Table 1
Concentration of calibrators (Cal 1–6) and quality controls (QCs)

Calibrator final conc. (ng/g)	QC final conc. (ng/g)
Cal 1 = 5.00	Negative QC = 0.00
Cal 2 = 10.0	Low QC = 6.00
Cal 3 = 25.0	Mid QC = 30.0
Cal 4 = 50.0	High QC = 165
Cal 5 = 100	
Cal 6 = 220	

2. Calibrator working solution 6: 4.40 μg/mL EtG. Add 880 μL of standard stock solution to 5 mL of methanol in a 10 mL Class A volumetric flask then fill to volume with methanol and mix well (*see* **Note 3** and Table 1).
3. Calibrator working solution 5: 2.00 μg/mL EtG. Add 2.5 mL of calibrator working solution 6 to 3.0 mL of methanol and mix well.
4. Calibrator working solution 4: 1.00 μg/mL EtG. Add 2.5 mL of calibrator working solution 5 to a 5 mL Class A volumetric flask. Fill to volume with methanol and mix well.
5. Calibrator working solution 3: 0.500 μg/mL EtG. Add 2.5 mL of calibrator working solution 4 to a 5 mL Class A volumetric flask. Fill to volume with methanol and mix well.
6. Calibrator working solution 2: 0.200 μg/mL EtG. Add 2.0 mL of calibrator working solution 3 to a 5 mL Class A volumetric flask. Fill to volume with methanol and mix well.
7. Calibrator working solution 1: 0.100 μg/mL EtG. Add 2.5 mL of calibrator working solution 2 to a 5 mL Class A volumetric flask. Fill to volume with methanol and mix well.

2.5 Quality Controls (QCs) and Internal Standard

1. QC standard stock solution: 50 μg/mL EtG in methanol. Add 500 μL of 1.0 mg/mL EtG (different lot number) to 5 mL of methanol in a 10 mL Class A volumetric flask. Fill to volume with methanol and mix well (*see* **Note 4**).
2. QC working solution 4: 3.30 μg/mL EtG. Add 660 μL of QC standard stock solution to 5 mL of methanol in a 10 mL Class A volumetric flask. Fill to volume with methanol and mix well (*see* **Note 5** and Table 1).
3. QC working solution 3: 0.600 μg/mL EtG. Add 1.0 mL of QC working solution 4 to 4.5 mL of methanol and mix well.

4. QC working solution 2: 0.120 μg/mL EtG. Add 1.0 mL of QC working solution 3 to a 5 mL Class A volumetric flask. Fill to volume with methanol and mix well.
5. QC working solution 1: 0.000 μg/mL EtG (blank). Methanol only.
6. Internal standard stock solution: 50 μg/mL EtG-d5 in methanol. Add 500 μL of 1.0 mg/mL Ethyl-β-D-glucuronide-d5 to 5 mL of methanol in a 10 mL Class A volumetric flask. Fill to volume with methanol and mix well (*see* **Note 6**).
7. Internal standard working solution: 0.500 μg/mL EtG-d5 in methanol. Add 100 μL of internal standard stock solution to 5 mL of methanol in a 10 mL Class A volumetric flask. Fill to volume with methanol and mix well.

2.6 Equipment and Instrumentation

1. Weighing balance.
2. Biotage® Bead Ruptor 24.
3. 48-place positive pressure manifold (*see* **Note 7**).
4. TurboVap® LV Concentration Evaporator.
5. AB SCIEX Triple Quad™5500 mass spectrometer interfaced with CTC PAL HTC-*xt*-DLW autosampler and Agilent 1260 Infinity series binary pump, degasser, and column oven, operated in negative electrospray ionization mode (*see* **Note 8**).
6. AB SCIEX Analyst® and MultiQuant™ software.

3 Methods

3.1 Sample Preparation

1. Label a series of 7.0 mL polypropylene screw cap tubes, a series of 16 × 100 mm, 14 mL culture tubes, and a series of autosampler vials with matching identifiers for calibrators, controls, and patient specimens (i.e., tubes 1–6 for calibrators, tubes 7–10 for the controls, and the other tubes for patient samples).
2. Using a clean razor blade, cut approximately 0.5 cm cubes of patient cord specimen (*see* **Note 9**).
3. Accurately weigh 1 ± 0.025 g of each patient cord specimen (0.975–1.025 g). Transfer to the appropriate pre-labeled 7.0 mL polypropylene screw cap tubes. Aliquot each patient cord specimen using a new, clean wooden stir stick for each specimen.
4. Accurately weigh 1 ± 0.025 g of drug-free umbilical cord (0.975–1.025 g) to pre-labeled 7.0 mL polypropylene screw cap tubes 1–10 for calibrators and controls (*see* **Note 10**).
5. Add 50 μL of the internal standard working solution to each tube (*see* **Note 11**). It is acceptable to use a repeating pipette.

6. Spike the calibrators.
   (a) Add 50 µL of the EtG calibrator working solution 1 to tube (calibrator) 1.
   (b) Add 50 µL of the EtG calibrator working solution 2 to tube (calibrator) 2.
   (c) Add 50 µL of the EtG calibrator working solution 3 to tube (calibrator) 3.
   (d) Add 50 µL of the EtG calibrator working solution 4 to tube (calibrator) 4.
   (e) Add 50 µL of the EtG calibrator working solution 5 to tube (calibrator) 5.
   (f) Add 50 µL of the EtG calibrator working solution 6 to tube (calibrator) 6.
7. Spike the controls.
   (a) Add 50 µL of the EtG QC working solution 1 to tube 7 (negative control) (*see* **Note 12**).
   (b) Add 50 µL of the EtG QC working solution 2 to tube 8 (low positive control).
   (c) Add 50 µL of the EtG QC working solution 3 to tube 9 (mid-positive control).
   (d) Add 50 µL of the EtG QC working solution 4 to tube 10 (high positive control).
8. Add 6 large UFO (5.6 mm) stainless steel beads to each tube.
9. Cap the tubes securely and place them in the Bead Ruptor 24.
10. Homogenize at 5.80 m/s for 4 cycles of 30 s, with 30 s pause in between cycles.
11. Remove samples from the Bead Ruptor 24, and verify that all specimens have a uniform appearance and no large pieces of cord are apparent. Samples will feel warm to the touch. Cool briefly before proceeding.
12. Remove the 6 large UFO (5.6 mm) stainless steel beads from each sample.
13. Centrifuge samples for 10 min at 0 °C and 20,598 × *g* (14,000 rpm). Remove samples from centrifuge carefully without disturbing the pellet.

3.2 Solid-Phase Extraction (SPE)

1. Place the SPE columns on a positive pressure manifold in the proper sequence in which they are labeled. SPE is by gravity flow only. Do not apply any pressure to the manifold.
2. Condition the SPE columns with 4 mL of methanol using gravity flow.

3. Equilibrate the SPE columns with 4 mL of ultrapure de-ionized water using gravity flow.
4. Load sample supernatants in proper sequence onto the SPE columns using gravity flow.
5. Wash columns (into a waste collection bin) with 3 mL of ultrapure de-ionized water using gravity flow.
6. Wash columns (into a waste collection bin) with 3 mL of methanol using gravity flow.
7. Dry the columns at 30–35 psi for 5 min on the positive pressure manifold.
8. Move SPE columns from the waste position to a collection tube rack with the pre-labeled 14 mL glass culture tubes.
9. Elute by gravity with 2 × 1.5 mL of freshly prepared elution solvent, into corresponding 14 mL glass culture tubes.
10. Place 14 mL glass culture tubes in the concentration evaporator at 40 °C and 20 psi for approximately 12 min. Evaporate the samples to dryness but do not dry for an extended period of time.
11. Add 1.0 mL of 0.1% formic acid in water (mobile phase A) to the dried extracts to reconstitute. Vortex mix the tubes briefly.
12. Transfer reconstituted samples from the glass culture tubes into corresponding pre-labeled autosampler vials, and cap each vial.
13. Dispose of waste and excess elution solvent into the waste container.
14. Load the vials on the autosampler for instrumental analysis.

3.3 Instrumental and Data Analysis

1. Flow rate: 0.350 mL/min.
2. Column temperature (°C): 25 ± 0.5.
3. LC gradient: *see* Table 2.
4. Autosampler settings: *see* Table 3.
5. Diverter valve settings: *see* Table 4.
6. Ion source settings: *see* Table 5.
7. Multiple reaction monitoring (MRM) transitions and MS parameters: *see* Table 6. The representative mass chromatograms for a calibrator and a patient sample are shown in Fig. 2.
8. Perform data analysis and quantitation with AB Sciex MultiQuant™ software according to the manufacturer's recommendations (*see* **Note 13**).
9. Construct calibration curve (*see* **Note 13**) using the six (non-zero) calibrators. Plot the averaged peak area ratios of EtG to internal standard (IS) against the nominal EtG

Table 2
LC gradient conditions

Total time (min)	A (%)	B (%)
0.00	100	0
0.20	99	1
3.00	99	1
3.03	20	80
3.05	2	98
4.05	2	98
4.06	100	0
5.00	100	0

Table 3
Autosampler parameters

Parameter	Value
Loop volume 1 (μL)	20
Loop volume 2 (μL)	20
Syringe type	100 μL DLW
Syringe volume (μL)	20
Injection volume (μL)	10.0
Cycle name	Analyst LC-Inj DLW Fast_Rev05
Airgap volume (μL)	3
Front volume (μL)	5
Rear volume (μL)	5
Filling speed (μL/s)	5
Pullup delay (ms)	3
Inject to	LC Vlv1
Injection speed (μL/s)	5
Pre inject delay (ms)	500
Post inject delay (ms)	500
Needle gap valve clean (mm)	3
Valve clean time solvent 2 (s)	3
Valve clean time solvent 1 (s)	3
Post clean time solvent 1 (s)	2

Table 4
Diverter valve parameters

Step	Diverter total time (min)	Column switching valve position[a]
1	0.00	A
2	2.00	B
3	3.20	A

[a]valve in position A = sample goes out to waste, valve in position B = samples go to MS

Table 5
MS source parameters

Parameter	Value
CUR (Curtain gas, psi)	35
CAD (Collisions gas, psi)	10
TEM (Temperature, °C)	600
GS1 (Nebulizer gas, psi)	50
GS2 (Heater gas, psi)	50
IS (IonSpray voltage, V)	−3500
Declustering potential (V)	−90
Dwell time (ms)	80
Resolution	Unit mass (Q1 and Q3)

Table 6
MRM transitions and MS compound-dependent parameters

Compound	Q1	Q3	Entrance potential (V)	Collision energy (V)	Exit potential (V)
EtG 1	221.2	85.1	−10	−22	−5
EtG 2	221.2	75.1	−15	−20	−10
EtG-d5 1	226.1	85.1	−7	−22	−5
EtG-d5 2	226.1	75.0	−13	−20	−9

concentrations in the calibrators, using linear regression with $1/x^2$ weighting.

10. Review calibration and QC data (*see* **Note 14**). Evaluate calibrator and QC peak integration, chromatography, and presence of internal standard (IS). Review the retention times and

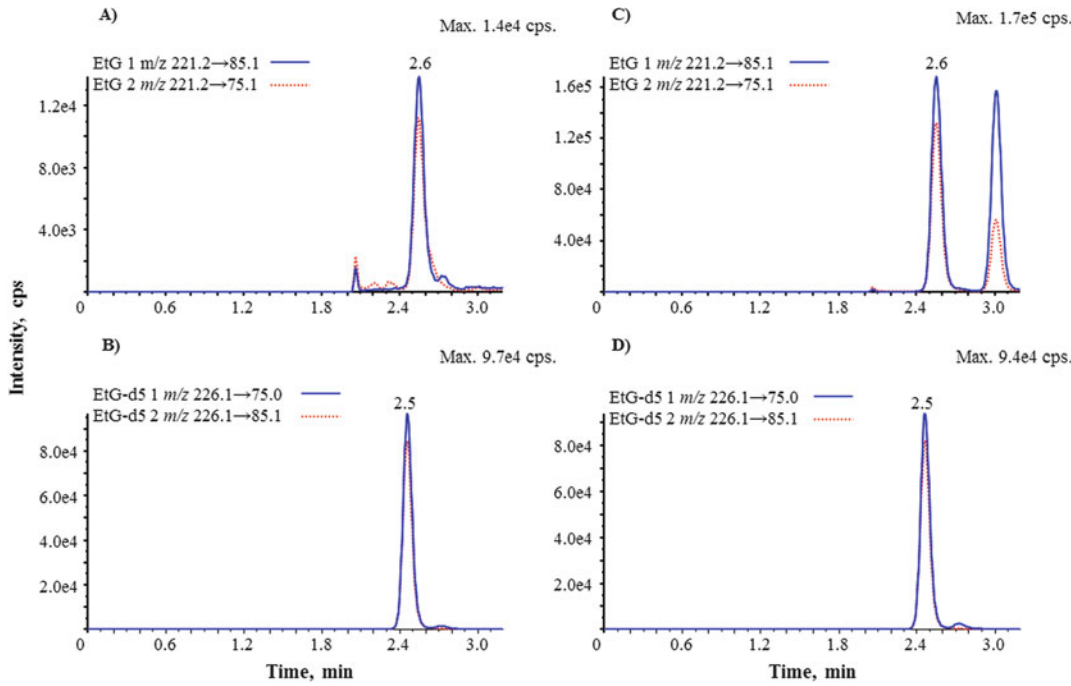

Fig. 2 The representative mass chromatograms for EtG and internal standard in (**a**) calibrator 1 (5 ng/g) and (**b**) internal standard (25 ng/g); (**c**) positive patient specimen (EtG = 62.9 ng/g) and (**d**) internal standard (25 ng/g)

ion mass ratios for EtG and IS for any flags. Verify the linear calibration range for the method (*see* **Note 15**).

11. Review patient data. For the patient data to be reported, all qualitative and quantitative criteria for the analyte and internal standard established must be met.

4 Notes

1. Potential carryover is dependent on the autosampler used; therefore, several autosampler wash solvents should be re-evaluated if a different autosampler is used to ensure there is no carryover specific to the LC-MS/MS system.
2. Caution should be used when collecting and preparing umbilical cord specimen for testing, to ensure that alcohol containing products are not used directly on specimen or nearby during sample processing. Post collection synthesis of EtG may cause false-positive results [16].
3. Calibrators are prepared by spiking an aliquot of drug-free human umbilical cord with the calibrator working solution prepared in methanol.

4. Quality control (QC) standards should be prepared from a second source (i.e., vendor/supplier or different lot number) than the calibrator standards.
5. Controls (negative, low, mid, and high QCs) are prepared by spiking an aliquot of drug-free human umbilical cord with the QC working solutions prepared in methanol.
6. Internal standard is necessary to correct for any variability throughout the analytical process; therefore, the internal standard selected should have similar properties as the analyte of interest. Ideally an isotopically labeled internal standard should be used as described here.
7. This method was validated using positive pressure for SPE extraction. The use of other (i.e., vacuum) manifolds was not evaluated in this work.
8. LC-MS/MS parameters will vary from one instrument or laboratory to another. The parameters described in this method can be used as starting conditions, but LC and MS parameters for the analyte and internal standard should be optimized for the specific instrument utilized.
9. Use a clean razor blade and weigh boat for each test specimen. Razors should never be reused. Discard razor blades in the sharps container and any biohazard waste appropriately.
10. Calibrators and controls should have the same matrix as the patient specimens and should be prepared in the same manner as the patient samples.
11. Every sample should be analyzed with the internal standard.
12. Negative control is a blank control that does not contain the analyte of interest, only drug-free umbilical cord tissue and the internal standard.
13. The MQ4 integration algorithm is used for peak integration. Integration parameters will need to be determined by the user based on the quality of the raw data to provide the most consistent and accurate peak integration. Linear regression with $1/x^2$ weighting (where x is the nominal concentration of a cord calibrator) is performed by the software, which establishes a linear regression calibration equation, from which the concentrations of the positive controls and the patient specimens are quantitatively determined and reported.
14. The calibrator and controls must have acceptable chromatography (i.e., peak shapes, peak areas) retention times (RT), and ion mass ratio (IMR) for the target analyte and internal standard. The concentration of the analyte in the six (non-zero) calibrators must be within $\pm 20\%$ of the target concentration (accuracy). The correlation coefficient (r) for the calibration curve generated must be ≥ 0.995. The negative control must

demonstrate the presence of the internal standard and should be negative for the (non-deuterated) analyte and meet all qualitative criteria. The positive (low, mid, and high) quality controls (QCs) should contain the reportable compound and the internal standard at the correct concentrations (i.e., should be within ±20% of the target concentration), and all must meet the established qualitative criteria for acceptance.

15. The limit of detection (LOD) for EtG in cord is 1 ng/g, the lower limit of quantitation (LLOQ) is 3.00 ng/g, and the upper limit of quantitation (ULOQ) is 220 ng/g. The linear calibration range of the method is 5.00–220 ng/g.

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Chapter 22

Analysis of Drugs in Oral Fluid Using LC-MS/MS

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Abstract

Oral fluid analysis for drugs is increasingly used in a variety of testing areas: pain management and medication monitoring, parole and probation situations, driving under the influence of drugs (DUID), therapeutic drug monitoring, and testing for drugs in the workplace. The sample collection itself is straightforward, rapid, observable, and noninvasive, requiring no special facilities (compared to urine) or medical personnel (compared to blood). The pH of saliva is slightly acidic relative to blood; therefore, drugs which are more basic tend to be present in higher concentration in oral fluid than in blood: cocaine, amphetamines, oxycodone, tramadol, buprenorphine, methadone, and fentanyl. Conversely, acidic drugs and drugs which are strongly protein bound have lower concentrations in oral fluid than in blood: examples include benzodiazepines, barbiturates, and carisoprodol. Because of the low volume of specimen available for analysis and the drug concentrations present (generally much lower than those in urine), efficient extraction methods and sensitive confirmation procedures are necessary for routine analysis of drugs in oral fluid. In this chapter, solid-phase extraction methods are described for a variety of drugs with liquid chromatography—tandem mass spectrometry detection.

Key words Oral fluid, Drugs, Solid-phase extraction, Hydrolysis, LC-MS/MS

1 Introduction

Oral fluid (saliva) consists of gingival crevicular fluid combined with secretions from the parotid gland (20–25%), the submandibular gland (70–75%), and other minor salivary glands. It is almost entirely water, but enzymes, cholesterol, proteins, and electrolytes are also present. A healthy individual will produce approximately 0.75–1 L per day with a pH value in the range 6.0–7.8. Advantages of using oral fluid as a test matrix include difficulty of adulteration (collection can be observed), speed and ease of collection, closer association with blood concentrations than urine, and potential for detection of recent intake [1–3]. While saliva can be collected neat via expectoration (i.e., "spitting"), generally a collection device consisting of a pad which is then transferred into a transportation buffer is used. The use of a pad improves the speed of recovery and

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indicates when adequate sample has been collected (in most devices) and the buffer assists with drug stability in transportation and storage; the analytical result can be affected by the mode of sample collection [4, 5]. In addition, collection devices have differing volumes of diluent (transportation buffer), so compensating for the dilution factor before reporting a quantitative result is also a necessary component of method development and validation.

In contrast to methods used for the analysis of drugs in urine, oral fluid procedures should include the parent drug (and metabolites), because the compound in highest concentration in saliva following drug use tends to be the parent drug.

Drug concentrations in oral fluid are also markedly lower than those detected in urine, so analytical procedures with increased sensitivity are required. In order to develop laboratory procedures using liquid chromatography—tandem mass spectrometry (LC-MS/MS) for drugs in oral fluid, knowledge of the components of any transportation buffer in the collected sample is also essential. Buffers containing non-ionic detergents (e.g., Tween®) should not be directly injected into LC-MS/MS instruments. In order to extend the life of chromatographic columns and components of the instrument as well as achieve the analytical sensitivity required for relevant detection of drugs in oral fluid, the analytes should be efficiently extracted from the matrix prior to injection and analysis. Methods of sample preparation for large drug panels include supported liquid extraction [6], liquid-liquid extraction [7], and solid-phase extraction [8].

In the following procedures, solid-phase extraction methods are described for a variety of drugs, with LC-MS/MS detection.

2 Materials

All solutions should be prepared with ultrapure water and high reagent grade salts. All solvents should be of high purity for extraction, reconstitution, and mobile phase preparation. Drug standards and deuterated compounds to be used as internal standards should be of high quality and commercially available (*see* **Note 1**). The volume of each solution can be scaled up as needed depending on the size of batch to be analyzed.

2.1 Supplies

1. Agilent 1200 Series LC pump, 6430 or 6410 LC-MS/MS, or similar, operating in electrospray ionization mode.
2. Agilent MassHunter software.
3. Eclipse Plus C18 4.6 mm × 50 mm 1.8 μm column or Eclipse Plus C18 2.1 mm × 50 mm 1.8 μm column.
4. Quantisal® oral fluid collection device (*see* **Note 2**).

5. CEREX Clin II extraction columns, 3 mL, 35 mg.
6. Trace-N extraction columns, 3 mL, 15 mg.
7. Positive pressure extraction manifold.
8. Sample concentrator operated under ultrahigh-purity nitrogen.
9. Heating block capable of temperatures up to 70 °C.
10. Vortexer.
11. Borosilicate glassware (*see* **Note 3**).
12. Amber borosilicate glass screw top vials.
13. Vacuum oven.
14. Borosilicate glass crimp top high recovery 30 µL reservoir autosampler vials.
15. Synthetic oral fluid, Immunalysis.
16. Borosilicate glass 9″ transfer pipettes.
17. Snap cap 11 mm plastic autosampler vials and caps.

2.2 Prepared Reagents for Basic Drugs

1. Conditioning buffer: 0.1 M sodium phosphate pH 6.0. Add 13.6 g of sodium phosphate to 1 L of deionized (DI) water and adjust pH 6.0 by adding ammonium hydroxide. Solution can be stored up to 6 months.
2. Wash solution: 0.1 M hydrochloric acid. Add 8.4 mL of hydrochloric acid to 1 L of DI water. Solution can be stored up to 6 months.
3. Extraction eluant: 78:20:2 methylene chloride/methanol/ammonium hydroxide. Mix 7.8 mL of methylene chloride, 2 mL of methanol, and 200 µL of ammonium hydroxide. Prepare fresh daily (*see* **Note 4**).
4. Mobile phase A: 20 mM ammonium formate: Add 1.26 g of ammonium formate to 1 L of HPLC grade water. No adjustment is necessary for the pH. Solution is stored up to 6 months.
5. Mobile phase B: HPLC grade methanol.
6. Reconstitution solution: 50:50 methanol/water. Mix 5 mL of methanol and 5 mL of water. Prepare fresh daily.
7. Benzoylecgonine (BZE) intermediate solution: 1000 ng/mL BZE. Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of 1 mg/mL BZE stock solution and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
8. 100 ng/mL BZE methanol spiking solution. Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 1000 ng/mL BZE intermediate solution. Bring to volume

with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.

9. Cocaine (COC) and cocaethylene (CE) intermediate solution: 1000 ng/mL COC and CE. Add 5 mL of acetonitrile to a 10 mL volumetric flask. Add 10 µL of each of the 1 mg/mL COC and CE stock solutions, and bring to volume with acetonitrile. Solution is stable for 6 months when stored at −20 °C.
10. 100 ng/mL COC and CE combined acetonitrile spiking solution: Add 5 mL of acetonitrile to a 10 mL volumetric flask. Add 1 mL of the 1000 ng/mL COC and CE intermediate solution. Bring up to volume with acetonitrile. Mix well. Solution is stable for 6 months when stored at −20 °C.
11. 100 ng/mL BZE-d_3 methanol internal standard spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of the 100 µg/mL BZE-d_3 stock solution, and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
12. 100 ng/mL COC-d_3 and CE-d_8 acetonitrile internal standard spiking solution: Add 5 mL of acetonitrile to a 10 mL volumetric flask. Add 10 µL each of the 100 µg/mL COC-d_3 and CE-d_8 stock solutions, and bring to volume with acetonitrile. Mix well. Solution is stable for 6 months when stored at −20 °C.
13. Quality controls are purchased from an outside vendor and should be within the linear range of the curve.

2.3 Prepared Reagents for Acidic/Neutral Drugs

1. 8 N sodium hydroxide (NaOH): Add 16 g of NaOH to 50 mL of DI water. Mix to dissolve. Solution is stable for 6 months when stored at room temperature.
2. Sample preparation solution: 0.1 M acetic acid pH 4.0. Add 5.7 mL of glacial acetic acid to 1 L of DI water, and adjust to pH 4.0 by adding drops of 8 N NaOH. Solution is stable for 6 months when stored at room temperature.
3. Conditioning solution: 0.1 M acetic acid. Add 5.7 mL of glacial acetic acid to 1 L of DI water. Mix well. Solution is stable for 6 months when stored at room temperature.
4. **Step 1** wash solution: 80:20 DI water/glacial acetic acid. Add 8 mL of DI water to a beaker. Add 2 mL of glacial acetic acid, stirring to mix. Prepare fresh daily.
5. **Step 2** wash solution: 40:60 DI water/methanol. Add 4 mL of DI water and 6 mL of methanol to a beaker and mix. Prepare fresh daily.
6. Extraction eluant: 98:2 hexane/glacial acidic acid. Add 9.8 mL of hexane to a beaker. Add 200 µL of glacial acetic acid, stirring to mix. Prepare fresh daily.

7. Mobile phase A: 5 mM ammonium formate. Add 0.315 g of ammonium formate to 1 L of HPLC grade water. Mix to dissolve. Solution is stored up to 6 months.
8. Mobile phase B: 0.5% formic acid in acetonitrile. Add 5 mL of formic acid to 1 L of acetonitrile. Mix well. Solution is stored up to 6 months.
9. Reconstitution solution: 50:50 5 mM ammonium formate:0.5% formic acid in acetonitrile. Add 5 mL of 5 mM ammonium formate to 5 mL of 0.5% formic acid. Prepare fresh daily.
10. Delta 9-tetrahydrocannabinol (THC), cannabinol (CBN), and cannabidiol (CBD) intermediate solution: 1000 ng/mL THC, CBN, and CBD. Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of each of the 1 mg/mL THC, CBN, and CBD stock solutions, and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
11. 100 ng/mL THC, CBN, and CBD combined spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 1000 ng/mL THC, CBN, and CBD intermediate solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
12. 10 ng/mL THC, CBN, and CBD methanol spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 100 ng/mL THC, CBN, and CBD spiking solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
13. 100 ng/mL THC-d_3 methanol internal standard spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of the 100 µg/mLTHC-d_3 stock solution and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
14. Quality controls are purchased from an outside vendor and should be within the linear range of the curve.

2.4 Prepared Reagents for Derivatization of Cannabinoids

1. 8 N NaOH: Add 16 g of NaOH to 50 mL of DI water. Mix to dissolve. Solution is stored at room temperature up to 6 months.
2. Base hydrolysis solution: 1 N NaOH. Dilute 1 mL of 8 N NaOH in 7 mL of DI water. Solution is prepared fresh daily.
3. Conditioning solution: 0.1 M acetic acid. Add 5.7 mL of glacial acetic acid to 1 L of DI water. Solution is stored at room temperature up to 6 months.
4. **Step 1** wash solution: 80:20 DI water/glacial acetic acid. Add 8 mL of DI water to a beaker. Add 2 mL of glacial acetic acid, stirring to mix. Prepare fresh daily.

5. **Step 2** wash solution: 40:60 DI water/methanol. Add 4 mL of DI water and 6 mL of methanol to a beaker and mix. Prepare fresh daily.
6. Extraction eluant: 98:2 hexane/glacial acidic acid. Add 9.8 mL of hexane to a beaker. Add 200 μL of glacial acetic acid, stirring to mix. Prepare fresh daily.
7. Derivatizing reagent catalyst: 10 mM triphenylphosphine (TPP). Add 0.2 g of TPP to 10 mL of acetonitrile. Mix to dissolve. Solution is stored at −20 °C up to 1 month in an amber borosilicate glass screw top vial.
8. Derivatizing reagent 1: 10 mM 2,2′-dithiodipyridine (DPDS). Add 0.2 g of DPDS to 10 mL of acetonitrile. Mix to dissolve. Solution is stored at −20 °C up to 1 month in an amber borosilicate glass screw top vial.
9. Derivatizing reagent 2: 10 μg 2-picolylamine (2-PA). Add 10 μL of 2-PA to 10 mL of acetonitrile and mix. Solution is stored at −20 °C up to 1 month in an amber borosilicate glass screw top vial.
10. Reconstitution solution: 50:50 HPLC water/acetonitrile. Add 5 mL of HPLC grade water and 5 mL of acetonitrile to a beaker. Mix well. Prepare fresh daily.
11. Mobile phase A: 5 mM ammonium formate. Add 0.315 g of ammonium formate to 1 L of HPLC grade water. Solution is stored up to 6 months.
12. Mobile phase B: 0.5% formic acid in acetonitrile. Add 5 mL of formic acid to 1 L of acetonitrile. Solution is stored up to 6 months.
13. THC, CBN, and CBD intermediate solution: 1000 ng/mL THC, CBN, and CBD. Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 μL of each of the 1 mg/mL THC, CBN, and CBD stock solutions, and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
14. 100 ng/mL THC, CBN, and CBD combined spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 1000 ng/mL THC, CBN, and CBD intermediate solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
15. 10 ng/mL THC, CBN, and CBD methanol spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 100 ng/mL THC, CBN, and CBD spiking solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.

16. 100 ng/mL THC-d_3 methanol internal standard spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of the 100 µg/mLTHC-d_3 stock solution and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
17. 100 ng/mL 11-nor-9-carboxy-delta-9-THC (THC-COOH) intermediate solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of the 100 µg/mL THC-COOH stock solution and bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
18. 2.5 ng/mL THC-COOH spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 25 µL of the 100 ng/mL THC-COOH intermediate solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
19. 0.25 ng/mL THC-COOH spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 2.5 ng/mL THC-COOH spiking solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
20. 100 ng/mL THC-COOH-d_9 intermediate solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 10 µL of the 100 µg/mL THC-COOH-d_9 stock solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
21. 2.5 ng/mL THC-COOH-d_9 internal standard spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 25 µL of the 100 ng/mL THC-COOH-d_9 intermediate solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
22. Quality controls are purchased from an outside vendor and should be within the linear range of the curve.

2.5 Prepared Reagents for Chiral Separation of Amphetamine and Methamphetamine

1. Conditioning buffer: 0.1 M sodium phosphate pH 6.0. Add 13.6 g of sodium phosphate to 1 L of DI water. Mix to dissolve. While stirring, adjust to pH 6.0 with ammonium hydroxide. Solution is stored up to 6 months.
2. Wash solution: 0.1 M hydrochloric acid. Add 8.4 mL of concentrated hydrochloric acid to 1 L of DI water. Solution is stored up to 6 months.
3. Extraction eluant: 98:2 methanol/ammonium hydroxide. Add 9.8 mL of methanol to a beaker. Add 200 µL of ammonium hydroxide and mix well. Eluant is prepared fresh daily.

4. Derivatizing reagent (0.3% Marfey's reagent): Add 0.001 g of Marfey's reagent to 10 mL of acetone. Solution is stable for 3 months.
5. Saturated sodium bicarbonate solution: Add sodium bicarbonate to 10 mL DI water to the point of saturation. Solution is stable for 3 months.
6. Sodium bicarbonate working solution: Add 1 mL of saturated sodium bicarbonate solution to 9 mL of water, and vortex to mix. Make fresh daily.
7. 1 N hydrochloric acid: Dilute 1 mL of hydrochloric acid in 11 mL of DI water. Solution is stable for 3 months.
8. Mobile phase A: 20 mM ammonium formate. Add 1.26 g of ammonium formate to 1 L of HPLC grade water. No adjustment is necessary for the pH. Solution is stored up to 6 months.
9. Amphetamine (AMP) and methamphetamine (METH) intermediate solution: 2500 ng/mL of each l- and d-isomer. Add 5 mL of methanol to a 10 mL volumetric flask. Add 25 μL of each 1 mg/mL stock solution of l- and d-AMP and l- and d-METH. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
10. 250 ng/mL AMP and METH spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 1 mL of the 2500 ng/mL AMP and METH intermediate solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
11. 250 ng/mL AMP-d_5 and METH-d_5 internal standard spiking solution: Add 5 mL of methanol to a 10 mL volumetric flask. Add 25 μL of each 100 μg/mL AMP-d_5 and METH-d_5 stock solution. Bring to volume with methanol. Mix well. Solution is stable for 6 months when stored at −20 °C.
12. Quality controls are purchased from an outside vendor and should be within the linear range of the curve.

3 Methods

All procedures may be carried out at room temperature in laboratory conditions with personnel wearing suitable protective clothing. Calibrators, controls, and samples are prepared in borosilicate glass test tubes on the day of extraction, in a 1:3 mixture of synthetic oral fluid and Quantisal® collection buffer. The volume of Quantisal® collected sample used in analysis is 1 mL, equivalent to 0.25 mL of neat oral fluid.

3.1 Extraction and Analysis of Basic Drugs

Basic drugs (e.g., amphetamines, cocaine, etc.) diffuse into saliva from the blood relatively easily. A procedure for the extraction of cocaine and its metabolites from oral fluid using a dilution factor of four is described below. The general method can be applied to other basic drug classes for extraction.

3.1.1 Preparation of Calibrators, Controls, and Samples

1. Label borosilicate glass test tubes for Calibrators 1–5, each quality control, and specimens.
2. Mix 2 mL of synthetic oral fluid with 6 mL of Quantisal® extraction buffer (or other buffers depending on collection device).
3. Pipette 1 mL of this mixture into a borosilicate glass test tube for each calibrator and control (unless using external QC) to be used.
4. Pipette 1 mL of each sample to be tested into its own borosilicate glass test tube.
5. Add 40 μL of each 100 ng/mL internal standard (BZE-d_3, COC-d_3, and CE-d_8) to each calibrator, control, and specimen.
6. Do not spike any drug standards into the tube labeled Calibrator 1. This is the negative standard.
7. Spike 10 μL of each 100 ng/mL drug standard (BZE and COC/CE) into the tube labeled Calibrator 2, to give a final concentration of 4 ng/mL.
8. Spike 20 μL of each 100 ng/mL drug standard (BZE and COC/CE) into the tube labeled Calibrator 3, to give a final concentration of 8 ng/mL.
9. Spike 40 μL of each 100 ng/mL drug standard (BZE and COC/CE) into the tube labeled Calibrator 4, to give a final concentration of 16 ng/mL.
10. Spike 80 μL of each 100 ng/mL drug standard (BZE and COC/CE) into the tube labeled Calibrator 5, to give a final concentration of 32 ng/mL (*see* **Note 5**).
11. Add 1 mL of 0.1 M potassium phosphate pH 6.0 to each tube as a buffer, and mix well (*see* **Note 6**).

3.1.2 Extraction

1. Use one 3 mL, 35 mg CEREX Clin II extraction column per calibrator, control, and sample. Place each column in the positive pressure manifold with the pressure setting no higher than 10 psi.
2. Condition the columns by adding 2 mL of methanol to each. Allow the methanol to flow through the column bed at a rate no greater than 1 mL/min or one drop at a time. Do not allow

the column bed to become dry once the conditioning has begun (*see* **Note 7**).

3. Add 2 mL of 0.1 M sodium phosphate buffer pH 6.0 to each column. Allow the pH 6.0 buffer to flow through the column bed at the same rate as the methanol. Do not allow the column to become dry before loading the sample.
4. Pour each calibrator, control, or sample onto the appropriate column. Allow the sample to load on the column bed without the use of positive pressure.
5. Once the samples have completely loaded, wash the columns with 2 mL of DI water allowing the water to flow at a rate of 1 mL/min. Positive pressure with nitrogen can be used up to a pressure of 10 psi (*see* **Note 8**).
6. Add 2 mL of 0.1 M hydrochloric acid wash solution, allowing to flow at the same rate as DI water (*see* **Note 9**).
7. Dry the columns under nitrogen using positive pressure at 30 psi for 1 min.
8. Remove from pressure and add 1 mL of methanol allowing to flow at 1 mL/min.
9. Add 1 mL of ethyl acetate, allowing to flow at 1 mL/min.
10. Dry the columns under nitrogen using positive pressure at 30 psi for 7 min.
11. Place clean appropriately labeled borosilicate glass tubes in extraction collection rack. Dry the tips of the extraction columns and place above the appropriate collection tube.
12. Add 2 mL of extraction eluant to elute basic drugs from calibrators, controls, and samples.
13. Place tubes in the sample concentrator, and evaporate the collected eluant under nitrogen (*see* **Notes 10** and **11**).
14. Remove dry extraction tubes and add 50 µL reconstitution solution. Vortex well.
15. Using disposable borosilicate glass transfer pipettes, transfer each sample to labeled autosampler vials and cap.

3.1.3 Analysis

1. Autosampler temperature: 7 °C.
2. Injection volume: 5 µL.
3. Column temperature: 60 °C.
4. Binary pump flow rate: 0.2 mL/min (*see* **Note 12**).
5. Gradient:

   0 min: 80% mobile phase B (MP-B)

   4 min: 30% MP-B

   5–8 min: hold at 80% MP-B (re-equilibration).

Table 1
MRM transitions for BZE, COC, CE, and their deuterated analogs

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (V)	Collision energy (V)
BZE-d_3	293.3	171.2	120	20
BZE	290.3	168.1	120	15
BZE	290.3	105.1	120	15
COC-d_3	307.3	185.3	120	20
COC	304.3	182.3	120	20
COC	304.3	82.3	120	25
CE-d_8	326.3	204.4	160	20
CE	318.3	196.4	120	25
CE	318.3	82.2	120	25

6. Total run time: 8 min (*see* **Note 13**).
7. MS/MS mode: positive (+) ESI multiple reaction monitoring (MRM) mode.
8. Nebulizer gas temperature: 350 °C.
9. Nebulizer gas flow: 10 L/min.
10. Nebulizer pressure: 40 psi.
11. Positive capillary voltage: 3500 V.
12. The MRM transitions for BZE, COC, and CE are shown in Table 1 (*see* **Note 14**).

3.2 Extraction and Analysis of Acid/Neutral Drugs

Acidic/neutral drugs (e.g., barbiturates, THC) do not accumulate well into oral fluid, but they are easily extracted and analyzed. Cannabis is the most widely used illicit drug; detection of the psychoactive component, THC, in oral fluid is useful for the identification of recent drug use. A procedure for the extraction of THC, CBN, and CBD from oral fluid using a dilution factor of four is described below. The general method can be applied to other acid/neutral drug classes for extraction; modification of the LC-MS/MS transitions would be necessary for other drug classes.

3.2.1 Preparation of Calibrators, Controls, and Samples

1. Label borosilicate glass test tubes for Calibrators 1–5, each quality control, and specimens.
2. Mix 2 mL of synthetic oral fluid with 6 mL of Quantisal® extraction buffer (or other buffers depending on collection device).

3. Pipette 1 mL of this mixture into a borosilicate glass test tube for each calibrator and control (unless using external QC) to be used.
4. Pipette 1 mL of each sample to be tested into its own borosilicate glass test tube.
5. Add 25 μL of 100 ng/mL internal standard (THC-d_3) to each calibrator, control, and specimen.
6. Do not spike any drug standards into the tube labeled Calibrator 1. This is the negative standard.
7. Spike 25 μL of the 10 ng/mL drug standard (THC, CBN, and CBD) into the tube labeled Calibrator 2, to give a final concentration of 1 ng/mL.
8. Spike 50 μL of the 10 ng/mL drug standard (THC, CBN, and CBD) into the tube labeled Calibrator 3, to give a final concentration of 2 ng/mL.
9. Spike 10 μL of the 100 ng/mL drug standard (THC, CBN, and CBD) into the tube labeled Calibrator 4, to give a final concentration of 4 ng/mL.
10. Spike 20 μL of the 100 ng/mL drug standard (THC, CBN, and CBD) into the tube labeled Calibrator 5, to give a final concentration of 8 ng/mL (*see* **Note 5**).
11. Add 1 mL of 0.1 M acetic acid pH 4.0 to each tube to buffer, and mix well.

3.2.2 Extraction

1. Use one 3 mL, 15 mg Trace-N extraction column per calibrator, control, and sample. Place each column in the positive pressure manifold with the pressure setting no higher than 10 psi.
2. Condition the columns by adding 500 μL of methanol to each one. Allow the methanol to flow through the column bed at a rate no greater than 1 mL/min or one drop at a time. Do not allow the column bed to become dry once the conditioning has begun (*see* **Note 7**).
3. Add 100 μL of 0.1 M acetic acid to each column. Allow the 0.1 M acetic acid to flow through the column bed at the same rate as the methanol. Do not allow the column to become dry before loading the sample.
4. Pour each calibrator, control, and sample onto the appropriate column. Allow the sample to load on the column bed without the use of positive pressure.
5. Once the samples have completely loaded, wash the columns with 1 mL of **Step 1** wash solution. Positive pressure with nitrogen can be used up to a pressure of 10 psi.

6. Add 1 mL of **Step 2** wash solution (*see* **Note 15**).
7. Dry the columns under nitrogen using positive pressure at 30 psi for 7 min.
8. Place clean appropriately labeled borosilicate glass tubes in extraction collection rack. Dry tips of extraction columns and place above the appropriate collection tube.
9. Add 1 mL of extraction eluant to elute acidic and neutral drugs from calibrators, controls, and samples.
10. Place tubes in the sample concentrator and evaporate the collected eluant under nitrogen (*see* **Note 10**).
11. Remove dry extraction tubes and reconstitute each tube with 50 µL of reconstitution solution. Vortex well.
12. Using disposable borosilicate glass transfer pipettes, transfer each sample to labeled plastic autosampler vials and cap.

3.2.3 Analysis

1. Autosampler: 7 °C.
2. Injection volume: 10 µL.
3. Column temperature: 60 °C.
4. Binary pump flow rate: 0.2 mL/min.
5. Gradient: (*see* **Note 16**).

   0–2 min: 70% mobile phase B (MP-B)

   4–6 min: 90% MP-B

   6–8 min: 70% MP-B (re-equilibration)
6. Total run time: 6 min.
7. MS/MS mode: positive (+) ESI multiple reaction monitoring (MRM) mode.
8. Nebulizer gas temperature: 300 °C.
9. Nebulizer gas flow: 10 L/min.
10. Nebulizer pressure: 35 psi.
11. Positive capillary voltage: 3500 V.
12. The MRM transitions for THC, CBN, and CBD are shown in Table 2 (*see* **Note 14**).

3.3 The Use of Derivatization in Extraction and Analysis of Specific Drugs and Drug Classes

Derivatization is unusual in LC-MS/MS procedures, but the improvement in sensitivity has allowed these assays to become routine [9]. Derivatization is a helpful tool that can either increase sensitivity for analytes that do not chromatograph well or can be used to separate chiral isomers that don't separate under normal conditions.

Table 2
MRM transitions for THC, CBN, and CBD and their deuterated analogs

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (V)	Collision energy (V)
THC-d_3	318.3	196.3	150	20
THC	315	193	120	20
THC	315	123	120	30
CBN	311.2	223.1	120	20
CBN	311.2	195.1	120	25
CBD	315	193	120	20
CBD	315	123	120	30

3.3.1 Preparation of Calibrators, Controls, and Samples for THC-COOH

The identification of THC-COOH, a metabolite of THC, is essential for differentiation of cannabis use from potential passive exposure. Unfortunately THC-COOH is present in oral fluid at concentrations approximately 1000 times lower than those of THC; in addition THC-COOH has been shown to be glucuronidated in oral fluid; therefore, a hydrolysis procedure was also incorporated to improve detection. The derivatization of THC-COOH does not interfere with the analysis of THC, CBN, and CBD, which do not derivatize.

1. Label borosilicate glass test tubes for Calibrators 1–7, each quality control, and specimens.
2. Mix 4 mL of synthetic oral fluid with 12 mL of Quantisal® extraction buffer (or other buffers depending on collection device).
3. Pipette 2 mL of this mixture into a borosilicate glass test tube for each calibrator and control (unless using external QC) to be used.
4. Pipette 2 mL of each sample to be tested into its own borosilicate glass test tube.
5. Add 25 μL each of the 100 ng/mL THC-d_3 and the 2.5 ng/mL THC-COOH-d_9 internal standard to each calibrator, control, and specimen.
6. Do not spike any drug standards into the tube labeled Calibrator 1. This is the negative standard.
7. Spike 5 μL of the 100 ng/mL drug standard (THC, CBN, and CBD) and into the tube labeled Calibrator 2, to give a final concentration of 1 ng/mL (*see* **Note 5**).
8. Spike 25 μL of the 100 ng/mL drug standard (THC, CBN, and CBD) into the tube labeled Calibrator 3, to give a final concentration of 5 ng/mL.

9. Spike 50 µL of the 100 ng/mL drug standard (THC, CBN, and CBD) and 20 µL of the 0.25 ng/mL THC-COOH drug standard into the tube labeled Calibrator 4, for final concentrations of 10 ng/mL and 10 pg/mL, respectively.
10. Spike 100 µL of each 100 ng/mL drug standard (THC, CBN, and CBD) and 40 µL of the 0.25 ng/mL THC-COOH drug standard into the tube labeled Calibrator 5, for final concentrations of 20 ng/mL and 20 pg/mL, respectively.
11. Spike 25 µL of each 1000 ng/mL drug standard (THC, CBN, and CBD) and 10 µL of the 2.5 ng/mL THC-COOH drug standard into the tube labeled Calibrator 6, for final concentrations of 50 ng/mL and 50 pg/mL, respectively.
12. Spike 50 µL of each 1000 ng/mL drug standard (THC, CBN, and CBD) and 20 µL of the 2.5 ng/mL THC-COOH drug standard into the tube labeled Calibrator 7, for final concentrations of 100 ng/mL and 100 pg/mL, respectively.
13. Add 0.2 mL of 1 N sodium hydroxide to each calibrator, control, and sample to hydrolyze prior to solid-phase extraction.
14. Cap and incubate in the heating block at 60 °C for 15 min.
15. Remove from heat and allow to cool to room temperature.
16. Add 0.5 mL of glacial acetic acid to each sample and mix well.

3.3.2 Extraction of THC-COOH

1. Use one 3 mL, 15 mg Trace-N extraction column per calibrator, control, and sample. Place each column in the positive pressure manifold with the pressure setting no higher than 10 psi.
2. Condition the columns by adding 500 µL of methanol to each. Allow the methanol to flow through the column bed at a rate no greater than 1 mL/min or one drop at a time. Do not allow the column bed to become dry once the conditioning has begun (*see* **Note 7**).
3. Add 100 µL of 0.1 M acetic acid to each column. Allow the 0.1 M acetic acid to flow through the column bed at the same rate as the methanol. Do not allow the column to become dry before loading the sample.
4. Pour each calibrator, control, or sample onto the appropriate column. Allow the sample to load on the column bed without the use of positive pressure.
5. Once the samples have completely loaded, wash the columns with 1 mL of **Step 1** wash solution, allowing the wash to flow at a rate of 1 mL/min. Positive pressure with nitrogen can be used up to a pressure of 10 psi.

6. Add 1 mL of S**tep 2** wash solution, allowing the wash to flow at a rate of 1 mL/min.
7. Dry the columns under nitrogen using positive pressure at 30 psi for 7 min.
8. Place clean appropriately labeled borosilicate glass autosampler high recovery reaction vials in extraction collection rack. Dry tips of extraction columns and place above the appropriate collection vial (*see* **Note 17**).
9. Add 1 mL of extraction eluant to elute THC-COOH, THC, CBN, and CBD from calibrators, controls, and samples.
10. Evaporate the collected eluant under nitrogen (*see* **Note 10**).
11. Remove dry extraction vials and derivatize.
12. To the dry extraction vials, add the following in order:
    (a) 20 µL of derivatizing reagent catalyst (TPP)
    (b) 20 µL of derivatizing reagent 1 (DPDS)
    (c) 20 µL of derivatizing reagent 2 (2-PA)
13. Crimp cap the vials, making sure each is tightly sealed.
14. Heat vials for 10 min at 60 °C.
15. Remove vials from heat and allow to cool to room temperature.
16. Remove caps and evaporate to dryness in a vacuum oven.
17. Reconstitute in 50 µL of reconstitution solution. Mix well.

3.3.3 Analysis of THC-COOH

1. Autosampler: 7 °C.
2. Injection volume: 20 µL.
3. Column temperature: 60 °C.
4. Binary pump flow rate: 0.2 mL/min.
5. Gradient:
   0–2 min: 70% mobile phase B (MP-B)
   4–6 min: 90% MP-B
   6–8 min: 70% MP-B (re-equilibration)
6. Total run time: 8 min.
7. MS/MS mode: positive (+) ESI multiple reaction monitoring (MRM) mode.
8. Nebulizer gas temperature: 300 °C.
9. Nebulizer gas flow: 10 L/min.
10. Nebulizer pressure: 35 psi.
11. Positive capillary voltage is held at 3500 V.
12. The MRM transitions for THC-COOH, THC, CBN, CBD are shown in Table 3 (*see* **Note 14**).

Table 3
MRM transitions for THC-COOH, THC, CBN, and CBD and their deuterated analogs

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (V)	Collision energy (V)
THC-COOH–d_9	444	336	140	20
THC-COOH	435	327	130	20
THC-COOH	435	299	130	25
THC-d_3	318.3	196.3	150	20
THC	315	193	120	20
THC	315	123	120	30
CBN	311.2	223.1	120	20
CBN	311.2	195.1	120	25
CBD	315	193	120	20
CBD	315	123	120	30

3.3.4 Preparation of Calibrators, Controls, and Samples for Chiral Separation of AMP and METH

The legal form of methamphetamine, levo-methamphetamine (l-METH), is present in over-the-counter medications, whereas the illicit form dextro-methamphetamine (d-METH) is more potent and more likely to be abused; in workplace drug testing programs, there is often a need to differentiate the two. Chiral separation is possible by treating the sample with Marfey's reagent. The resulting chiral derivatives allow chromatographic separation of the enantiomers of both amphetamine (AMP) and methamphetamine (METH) [10].

1. Label borosilicate glass test tubes for Calibrators 1–6, each quality control, and specimens.
2. Mix 2 mL of synthetic oral fluid with 6 mL of Quantisal® extraction buffer (or other buffers depending on collection device).
3. Pipette 1 mL of this mixture into a borosilicate glass test tube for each calibrator and control (unless using external QC) to be used.
4. Pipette 1 mL of each sample to be tested into its own borosilicate glass test tube.
5. Add 50 μL of each 250 ng/mL internal standard (AMP-d_5 and METH-d_5) to each calibrator, control, and specimen.
6. Do not spike any drug standards into the tube labeled Calibrator 1. This is the negative standard.

7. Spike 10 µL of 250 ng/mL drug standard (AMP and METH) into the tube labeled Calibrator 2, to give a final concentration of 10 ng/mL (*see* **Note 5**).
8. Spike 25 µL of each 250 ng/mL drug standard (AMP and METH) into the tube labeled Calibrator 3, to give a final concentration of 25 ng/mL.
9. Spike 50 µL of each 250 ng/mL drug standard (AMP and METH) into the tube labeled Calibrator 4, to give a final concentration of 50 ng/mL.
10. Spike 100 µL of each 250 ng/mL drug standard (AMP and METH) into the tube labeled Calibrator 5, to give a final concentration of 100 ng/mL.
11. Spike 200 µL of each 250 ng/mL drug standard (AMP and METH) into the tube labeled Calibrator 6, to give a final concentration of 200 ng/mL.
12. Add 1 mL of 0.1 M potassium phosphate pH 6.0 to each tube to buffer, and mix well.

3.3.5 Extraction for Chiral Separation of AMP and METH

1. Use one 3 mL, 35 mg CEREX Clin II extraction column per calibrator, control, and sample. Place each column in the positive pressure manifold with the pressure setting no higher than 10 psi.
2. Condition the columns by adding 2 mL methanol to each. Allow the methanol to flow through the column bed at a rate no greater than 1 mL/min or one drop a time. Do not allow the column bed to become dry once the conditioning has begun (*see* **Note 7**).
3. Add 2 mL of 0.1 M sodium phosphate buffer pH 6.0 to each column. Allow the buffer to flow through the column bed at the same rate as the methanol. Do not allow the column to become dry before loading the sample.
4. Pour each calibrator, control, or sample onto the appropriate column. Allow the sample to load on the column bed without the use of positive pressure.
5. Once the samples have completely loaded, wash the columns with 2 mL of DI water allowing the water to flow at a rate of 1 mL/min. Positive pressure with nitrogen can be used up to a pressure of 10 psi.
6. Add 2 mL of 0.1 M hydrochloric acid to each column, allowing to flow at the same rate as DI water.
7. Dry the columns under nitrogen using positive pressure at 30 psi for 1 min.

8. Remove from pressure and add 1 mL of methanol allowing to flow at 1 mL/min.
9. Dry the columns under nitrogen using positive pressure at 30 psi for 7 min.
10. Place clean appropriately labeled borosilicate glass tubes in extraction collection rack. Dry tips of extraction columns and place above the appropriate collection tube.
11. Add 2 mL of extraction eluant to elute amphetamines from calibrators, controls, and samples.
12. Place tubes in the sample concentrator and evaporate the collected eluant under nitrogen (*see* **Note 10**).
13. Remove dry extraction tubes and derivatize.
14. Add 50 µL of prepared Marfey's reagent and 10 µL of a 1:10 dilution of the saturated sodium bicarbonate solution. Cap.
15. Heat tubes 30 min at 50 °C.
16. Allow tubes to cool.
17. Add 10 µL of 1 N hydrochloric acid to each tube. Vortex and transfer to autosampler vials. Cap.

3.3.6 Analysis for Chiral Separation of AMP and METH

1. Autosampler: 7 °C.
2. Injection volume: 5 µL.
3. Column temperature: 40 °C.
4. Binary pump flow: 0.1 mL/min.
5. Gradient:

   0 min: 50% MP-B

   10 min: 95% MP-B

   15–29 min: hold at 50% MP-B.
6. Total run time: 29 min.
7. MS/MS mode: positive (+) ESI multiple reaction monitoring (MRM) mode.
8. Nebulizer gas: 350 °C.
9. Nebulizer gas: flow 10 L/min.
10. Nebulizer pressure: 40 psi.
11. Positive capillary voltage: 4000 V.
12. The MRM transitions for AMP and METH are shown in Table 4 (*see* **Notes 14** and **18**).

Table 4
MRM transitions for AMP and METH and their deuterated analogs

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (V)	Collision energy (V)
AMP-d_5	393.5	96	80	10
AMP	388.5	119	80	10
AMP	388.5	91	80	35
METH-d_5	407.5	92	80	40
METH	402.4	119	80	10
METH	402.4	91	80	30

4 Notes

1. The Agilent Technologies Compendium [11] contains information on extraction and LC-MS/MS transitions for the following drugs in oral fluid:

   Benzodiazepines: alprazolam, bromazepam, chlordiazepoxide, clonazepam, diazepam, estazolam, flurazepam, lorazepam, midazolam, nordiazepam, oxazepam, phenazepam, temazepam, triazolam

   Stimulants: Cocaine, benzoylecgonine, cocaethylene, norcocaine, amphetamine, methamphetamine, MDMA, MDA, MDEA, methylphenidate, phentermine

   Opiates and opioids: Codeine, morphine, 6-acetylcodeine, 6-acetylmorphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, buprenorphine, fentanyl, methadone, EDDP, meperidine, propoxyphene, tapentadol, tramadol

   Cannabinoids and synthetic cannabinoids: THC, CP 47,497, CP 47,497-C8, HU-210, JWH-018, JWH-073, JWH-200, JWH-250, AM-2201

   Dissociative drugs: Dextromethorphan, ketamine, phencyclidine.

   Sleep aids: Zaleplon, zolpidem, zopiclone

   Antihistamines: Diphenhydramine, doxylamine

   Muscle relaxants: Carisoprodol, meprobamate cyclobenzaprine

   Antidepressants: Amitriptyline, amoxapine, citalopram, clomipramine, desipramine, dothiepin, doxepin, fluoxetine, fluvoxamine, imipramine, mianserin, mirtazapine, nortriptyline, paroxetine, protriptyline, sertraline, trazodone, trimipramine, venlafaxine

Barbiturates: Butalbital, pentobarbital, phenobarbital, secobarbital

2. Depending upon the method of collection for oral fluid, incorporation of a dilution factor may be necessary for quantitation. Neat oral fluid samples which are collected by expectoration ("spitting") into glass vials may need to be centrifuged prior to extraction to allow any sediment to settle. Most oral fluid samples are collected using a device which incorporates a pad which is placed into the mouth; there is likely to be a blue or yellow dye indication of adequate saliva absorption; then the pad is placed in a known amount of buffer to assist drug recovery and stability during transportation. The dilution factor of 4 used in these procedures is derived from the collection of oral fluid (1 mL) using the Quantisal® device which is then placed into transportation buffer (3 mL). Other collection devices may have different dilution factors which must be calculated for accurate quantitation.
3. Only borosilicate glassware should be used in the preparation of THC and THC-COOH specimens and spiking solutions.
4. Laboratory personnel are reducing or eliminating the use of methylene chloride because of carcinogenic concerns; however benzoylecgonine recovery is increased when methylene chloride is used instead of ethyl acetate.
5. The calibration curve consists of 5–7 points including the negative standard. Additional calibration levels can be incorporated if high concentrations of drug are suspected.
6. Meperidine (*see* Subheading 3.1): Most basic drugs can be loaded onto the extraction columns using a pH 6.0 buffer; however, meperidine requires a slightly more acidic environment (pH 4.0) to load efficiently. 0.1 M acetic acid adjusted to pH 4.0 can be used for both loading and washing purposes.
7. The methanol opens up binding sites in the column packing. The buffer adjusts the pH of the column to improve binding of the analytes to the column bed.
8. Carisoprodol (*see* Subheading 3.1): In order to increase carisoprodol recovery from oral fluid, replacing the last wash steps with methanol and water mixed at a volume ratio of 25:75 wash followed by hexane (1 mL) has been shown to optimally recover the drug. Elute carisoprodol and its metabolite, meprobamate, with 3 mL ethyl acetate and hexane mixed at a volume ratio of 50:50 [11].
9. Amphetamine (*see* Subheading 3.1): When washing the solid-phase columns, a stronger acid is preferred (hydrochloric .vs. acetic) to improve recovery; do not use ethyl acetate as part of the wash. Adding 0.035 N sulfuric acid (10 μL) to the eluant

during evaporation assists the volatile amphetamines to stay in solution. Sulfuric acid cannot be used for chiral separation, which requires a basic environment.

10. Use a temperature no higher than 40 °C and a flow rate that only flutters the surface of the eluant. This drying flow rate will differ greatly based on make and model of evaporator and how many rows of tubes are drying. Do not allow samples to remain under drying conditions after they have completed evaporation.
11. Phencyclidine (PCP) (*see* Subheading 3.1): To increase the solid-phase extraction recovery of PCP in oral fluid, dry down samples at ambient temperature; do not over dry samples while under nitrogen.
12. The flow rate of 0.2 mL/min on the larger bore LC column is low, but is necessary to separate benzoylecgonine from norcocaine as they share the same transitions. While norcocaine is not included in this method, it is possible that its detection is useful for other biological matrices.
13. Opiates (*see* Subheading 3.1): Some opioids and metabolites share transitions in MS/MS mode (e.g., hydrocodone and codeine, noroxycodone and oxymorphone, etc.). Therefore, chromatographic separation is critical to correct identification and quantitation [12].
14. Transition ratios are calculated using the quantifying and qualifying ion transitions at the cutoff concentrations; subsequently the established ratio must be met (±20%) in order for a sample to be certified as positive. The use of a second qualifying transition ion and establishment of a ratio between quantifying and qualifying transitions intensities gives greater analyte certainty when identifying a positive sample. Analytes can have similar transitions and retention times, but the inclusion of the second transition and the transition ratio can be used to differentiate one analyte from another.
15. Barbiturates (*see* Subheading 3.2): Reducing the amount of methanol used in washing the columns to below 25% of the organic phase increases recovery, e.g., methanol/DI water volume ratio 25:75.
16. Benzodiazepines (*see* Subheading 3.2): An isocratic pump program of 20 mM ammonium formate pH 8.6: methanol (50:50) allows for good separation of numerous benzodiazepines. Deuterated clonazepam-d4 interferes with the chromatography of lorazepam; if this is an issue, consider removal or use a different internal standard.
17. Cannabinoids (*see* Subheadings 3.2 and 3.3.1): Using high recovery 1.5 mL reservoir vials greatly increased recovery of

THC-COOH. The vials are large enough to directly elute the drugs and have a tapered reservoir that holds the derivatizing reagents in place. The contents of the vial can then be evaporated and reconstituted in situ, eliminating loss in transfer steps.

18. Because the l- and d-isomers have the same transitions and same transition ratios, identification of the l- and d-isomer for both AMP and METH is based on retention time. The l-isomer chromatographs first with the d-isomer separated by approximately 0.3 min downstream.

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Chapter 23

Determination of Cocaine and Metabolites in Dried Blood Spots by LC-MS/MS

Lars Ambach and Christophe Stove

Abstract

Cocaine is one of the most common illegal drugs in Europe and other parts of the world. It is mainly metabolized to benzoylecgonine and ecgonine methyl ester but also to minor metabolites such as norcocaine and *meta*-hydroxy-benzoylecgonine. If ethanol is consumed simultaneously, cocaethylene is formed. Dried blood spots (DBS) are a minimally invasive sampling technique producing easy-to-ship specimens and have garnered increasing attention in forensic and clinical contexts in recent years. We hereby present a liquid chromatography/tandem mass spectrometry-based (LC-MS/MS) method for the quantification of cocaine, benzoylecgonine, ecgonine methyl ester, norcocaine, *meta*-hydroxy-benzoylecgonine, and cocaethylene in DBS.

Key words Dried blood spots, Cocaine, Benzoylecgonine, Ecgonine methyl ester, Norcocaine, Cocaethylene, Meta-hydroxy-benzoylecgonine, HPLC-MS/MS

1 Introduction

Cocaine (COC) is an illicit stimulant of the central nervous system and the most common illegal drug in Europe after cannabis [1]. It is the most frequently found stimulant in Southern and Western Europe with evidence suggesting an increasing use in recent years [1, 2].

In vivo, COC is rapidly converted into benzoylecgonine (BE) and ecgonine methyl ester (EME) by cleavage of one of the respective ester moieties. BE can be formed by carboxylesterases in the liver or through spontaneous hydrolysis at alkaline or physiological pH. EME can be formed in vivo and in vitro. *N*-demethylation to norcocaine (nor-COC) and hydroxylation of BE at the phenyl ring have been described as minor metabolic steps [3]. *Meta*-hydroxy-benzoylecgonine (*m*-OH-BE) has a longer half-life than BE [4, 5] and is formed exclusively in vivo [6]. It can therefore also be used to verify positive COC results if an

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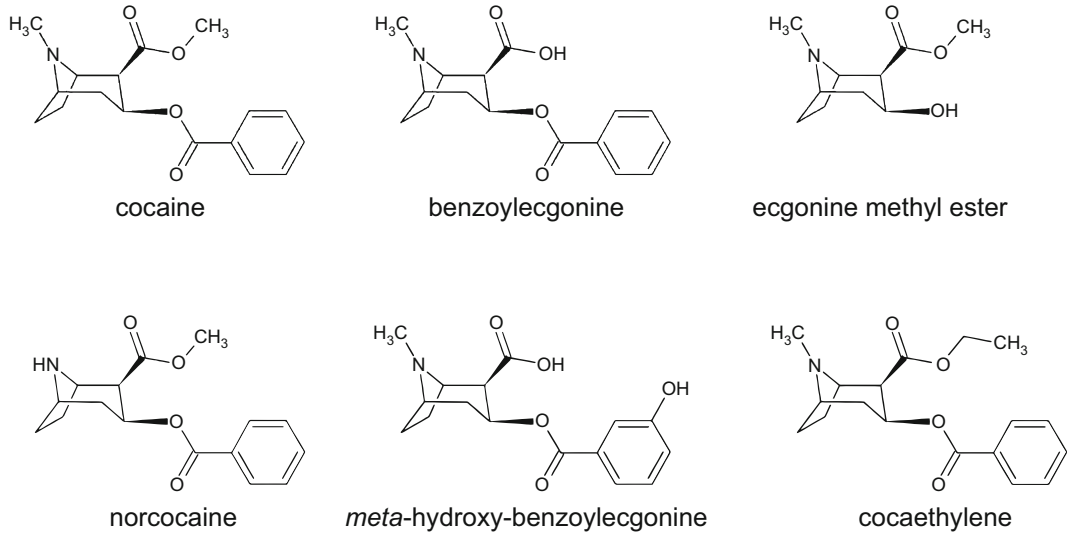

Fig. 1 Structures of cocaine and the included metabolites

external contamination with COC followed by spontaneous hydrolysis to BE is alleged. When ethanol (EtOH) is co-consumed with COC, cocaethylene (COC-Et) is produced through transesterification. An overview of the different structures is given in Fig. 1.

Dried blood spots (DBS) are generally produced by spotting capillary or venous blood onto filter paper, followed by drying, extraction, and analysis. DBS provide the opportunity for minimally invasive sampling with only small sample volumes required. For neonatal metabolic screening, DBS from capillary blood obtained from heel pricks have been used for decades [7], whereas for adults, blood obtained from the fingertip by lancet puncture is commonly used [8, 9]. Venous blood can also be used; in this case, DBS serve as a means of sample preparation for small sample volumes [10, 11]. DBS have been used in therapeutic drug monitoring [8, 12] as well as for the detection of drugs of abuse, medications, and their metabolites [13–17].

One of the most prominent challenges associated with DBS is the influence of the hematocrit (HCT) on analytical results. Due to higher viscosity at higher HCT, samples with different HCT will form spots of different sizes. Consequently, sub-punches of these spots will then represent different volumes of blood, leading to different quantitative results. Various strategies to overcome this challenge have been discussed [18].

Several methods for the quantification of COC in dried matrices have already been published. However, most of these cover a maximum of 2–3 metabolites [19–23]. We hereby present a more comprehensive, sensitive method for the quantification of COC and five of its metabolites (BE, EME, nor-COC, *m*-OH-BE, and COC-Et), which also enables analysts to assess co-consumption of EtOH (through COC-Et) and verify sample authenticity (through *m*-OH-BE).

2 Materials

2.1 Prepared Reagents

All solvents and reagents are of LC-MS or analytical grade.

1. Stock solutions: 1 mg/mL COC, nor-COC, COC-Et, *m*-OH-BE; 0.1 mg/mL BE, EME, and internal standards (IS) cocaine-D_3, benzoylecgonine-D_8, ecgonine methyl ester-D_3, norcocaine-D_3, cocaethylene-D_8 reference standards in sealed glass ampoules. Open, and transfer the contents of the ampoules into glass vials with a plastic screw cap and rubber/Teflon septum, and wrap in parafilm. Store vials at −20 °C.
2. Blank blood for calibration and quality control (QC) samples: Obtain blood from healthy volunteers using collection tubes with sodium fluoride and potassium oxalate (NaF/K_2Ox). Simultaneously collect one ethylenediaminetetraacetic acid (EDTA) tube of blood and determine the HCT value on a standard clinical analyzer. Based on the measured HCT, adjust the HCT of the NaF/K_2Ox-stabilized blood to 40% by adding or removing plasma to or from the blood (*see* **Notes 1** and **2**).
3. Extraction solvent: 6 ng/mL of each IS in 2 mM ammonium acetate. Dissolve 15.42 mg of ammonium acetate in 100 mL of ultrapure water (resistivity >18 MΩ cm). Add 3 μL of each IS stock solution to 50 mL of 2 mM ammonium acetate solution. Store at −20 °C (*see* **Note 3**).
4. Mobile phase A: 10 mM ammonium formate with 0.1% formic acid. Dissolve 630.6 mg of ammonium formate in 1 L of ultrapure water. Add 1000 μL of formic acid. Store at room temperature. Prepare freshly every week.
5. Mobile phase B: Methanol (MeOH) with 0.1% formic acid. Add 1000 μL of formic acid to 1 L of MeOH.
6. Stock blood for calibration samples: 1000 ng/mL of each analyte. Mix 2928 μL of blank blood with 3 μL of each of the 1.0 mg/mL reference solutions of COC, nor-COC, COC-Et, and *m*-OH-BE and 30 μL of each of the 0.1 mg/mL reference solutions of BE and EME. Stock blood also serves as Calibrator 7.
7. Calibration samples 1–6: Dilute stock blood with blank blood according to the scheme laid out in Table 1.
8. Stock blood for quality control (QC) samples: 1000 ng/mL of each analyte. Mix 2928 μL of blank blood with 3 μL of each of the 1.0 mg/mL reference solutions of COC, nor-COC, COC-Et, and *m*-OH-BE and 30 μL of each of the 0.1 mg/mL reference solutions of BE and EME.
9. QC samples 1–4: Dilute stock blood with blank blood according to the scheme laid out in Table 2.

Table 1
Dilution scheme for calibration samples

Calibrator	C7	C6	C5	C4	C3	C2	C1
Volume blank blood added (μL)	–	300	630	570	630	570	540
Volume stock blood added (μL)	600	300	70	30	–	–	–
Volume C5 added (μL)	–	–	–	–	70	30	–
Volume C3 added (μL)	–	–	–	–	–	–	60
Final concentration (ng/mL)	1000	500	100	50	10	5.0	1.0

Table 2
Dilution scheme for quality control (QC) samples

QC sample	QC IV	QC III	QC II	QC I
Volume blank blood added (μL)	150	555	540	580
Volume stock blood added (μL)	450	45	–	–
Volume QCIII added (μL)	–	–	60	20
Final concentration (ng/mL)	750	75	7.5	2.5

2.2 Supplies and Analytical Equipment

1. Blood collection tubes containing NaF/K_2Ox or EDTA (*see* **Note 4**).
2. Whatman® 903 Specimen Collection Paper.
3. Eppendorf 2 mL plastic microcentrifuge tubes or similar.
4. Shaker for 2 mL plastic microcentrifuge tubes, e.g., Biosan TS-100C Thermo-Shaker.
5. Centrifuge with rotors for microcentrifuge tubes and blood collection tubes, e.g., Eppendorf Centrifuge 5804R.
6. Sample concentrator, e.g., TurboVap LV or Techne Dri-Block DB-3D.
7. Standard clinical analyzer capable of measuring hematocrit (HCT).
8. HPLC system capable of gradient elution and equipped with an autosampler and column oven, e.g., Shimadzu Prominence series.
9. Analytical column: Phenomenex Synergi Polar-RP, 100 × 2.0 mm, 2.5 μm.
10. Triple quadrupole mass spectrometer with electrospray ionization source, e.g., SCIEX QTRAP 5500 or similar.

3 Methods

3.1 Preparation of DBS for Calibrators and Controls

1. Take up 25 μL of Calibrator 1 with a pipette.
2. Transfer the blood in a single drop onto the specimen collection paper or collection card. The pipette tip should not touch the paper (*see* **Notes 5** and **6**).
3. Repeat **steps 1** and **2** for all calibrators, QCs, and blank blood.
4. Let the DBS dry at room temperature for 2 h (*see* **Notes 7** and **8**).

3.2 Extraction

1. Take a 6 mm punch from the center of each DBS (*see* **Note 9**).
2. Transfer each punch into its own 2 mL Eppendorf cup.
3. Add 200 μL of extraction solvent to each tube.
4. Cap and shake for 15 min at 1000 rpm/1.12 × *g* at room temperature.
5. Add 1000 μL of ice-cold acetonitrile (MeCN) (*see* **Note 10**).
6. Cap and shake for 10 min at 1000 rpm at room temperature.
7. Centrifuge the samples for 20 min at 14,000 × *g*, 4 °C.
8. Transfer the supernatants into labelled glass tubes or vials.
9. Evaporate the supernatants under a stream of nitrogen at 40 °C (*see* **Note 11**).
10. Reconstitute in 50 μL of mobile phase A (*see* **Note 12**).
11. Transfer to a labelled autosampler vial with a 100 μL insert.

3.3 Analysis

1. Place samples in the autosampler in the following order:
   (a) Calibration samples (including blank samples), lowest to highest concentration.
   (b) QC samples, lowest to highest concentration.
   (c) Unknown samples.
   (d) Solvent blank samples (*see* **Note 13**).
2. Set the LC-MS/MS acquisition method to run with the following parameters:
   (a) Autosampler parameters: Cool down to 4 °C, injection volume: 10 μL.
   (b) HPLC parameters (*see* **Note 14**):
   - Column temperature: 50 °C.
   - Flow rate: 0.3 mL/min.
   - Gradient program:

     0.0–0.5 min: Hold at 0% B.

     0.5–0.6 min: Increase linearly to 45% B.

0.6–3.0 min: Increase linearly to 95% B.

3.0–4.0 min: Hold at 95% B.

4.0–4.01 min: Decrease linearly to 0% B.

4.01–6.0 min: Hold at 0% B to re-equilibrate.

(c) Ion source parameters:

- Ion spray voltage: 5500 V.
- Source temperature: 500 °C.
- Ion source gas 1: 60 psi.
- Ion source gas 2: 40 psi.
- Curtain gas: 20 psi.

(d) MS parameters:

Compound-dependent parameters are listed in Table 3 (*see* **Notes 15** and **16**).

3. A chromatogram of a QC sample at the lower limit of quantitation (LLOQ) of EME ($c = 5.0$ ng/mL, highest LLOQ of all analytes) is shown in Fig. 2.

Table 3
Lower limits of quantification (LLOQ) and MRM transitions and parameters

Analyte	LLOQ (ng/mL)	*m/z* Q1	*m/z* Q3	Dwell time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Cocaine	2.5	304.3	82.0/150.0	30	46	10	39/33	12
Benzoylecgonine	1.0	290.1	82.0/168.1/150.1	30	70	10	37/27/32	10
Ecgonine methyl ester	5.0	200.1	82.0/182.1	30	45	10	32/23	9
Norcocaine	2.5	290.2	136.1/168.1/108.0	30	70	10	30/22/44	11
Cocaethylene	2.5	318.2	82.0/150.1	30	70	10	37/33	10
m-OH-benzoylecgonine	2.5	306.3	121.1/82.0	30	70	10	37/36	7
Cocaine-D_3	n/a	307.2	185.2	30	70	9	28	10
Benzoylecgonine-D_8	n/a	298.3	171.1	30	60	9	27	10
Ecgonine methyl ester-D_3	n/a	203.3	185.2	30	70	10	24	10
Norcocaine-D_3	n/a	293.2	136.1	30	57	10	30	10
Cocaethylene-D_8	n/a	326.3	204.2	30	70	10	28	12

Q1 quadrupole 1, *Q3* quadrupole 3, the first transition is used as quantifier, the following transitions as qualifiers, *DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential

4 Notes

1. The HCT of the calibration and QC samples should be approximately the (expected) median of the HCT of the unknown samples to be analyzed in order to minimize the influence of the HCT effect on the analytical result. The choice of the HCT of the blood in which calibrators and QC samples are prepared depends on the sample population. The HCT effect is compound-dependent. It is thus essential that the HCT effect

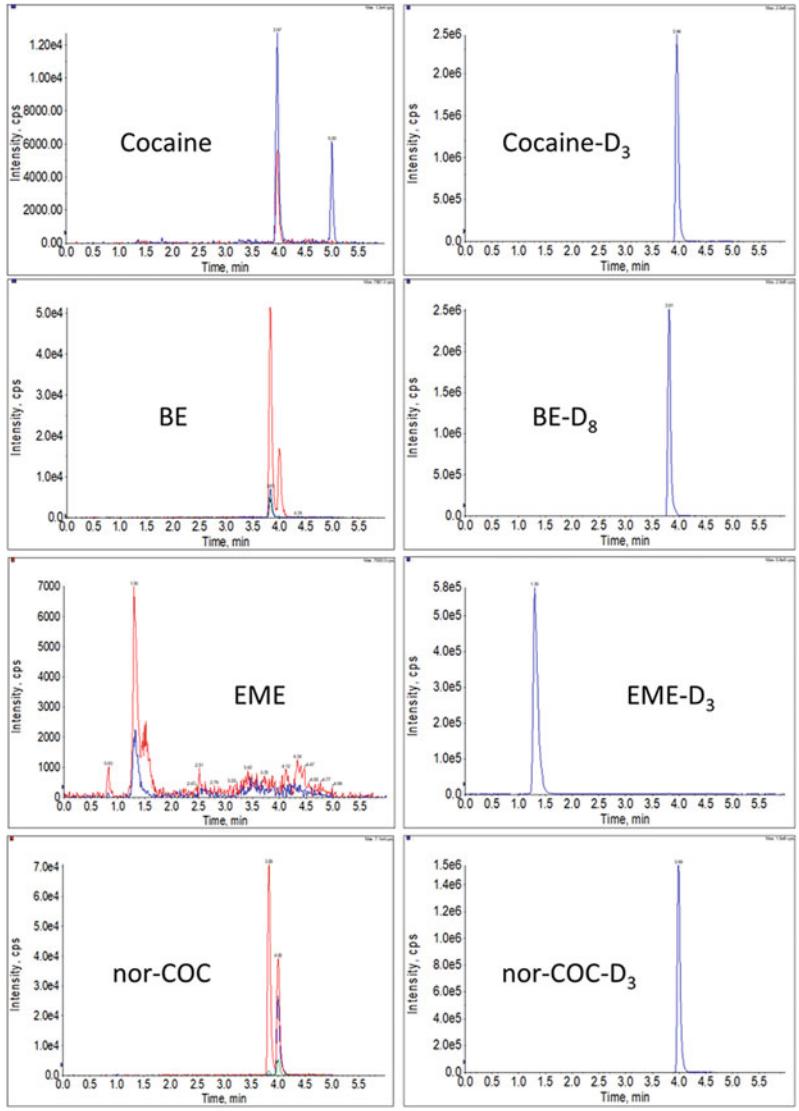

Fig. 2 Extracted ion chromatograms for all analytes and internal standards at the lower limit of quantitation of EME (5.0 ng/mL). Blue = qualifier transition, red/green = quantifier transitions

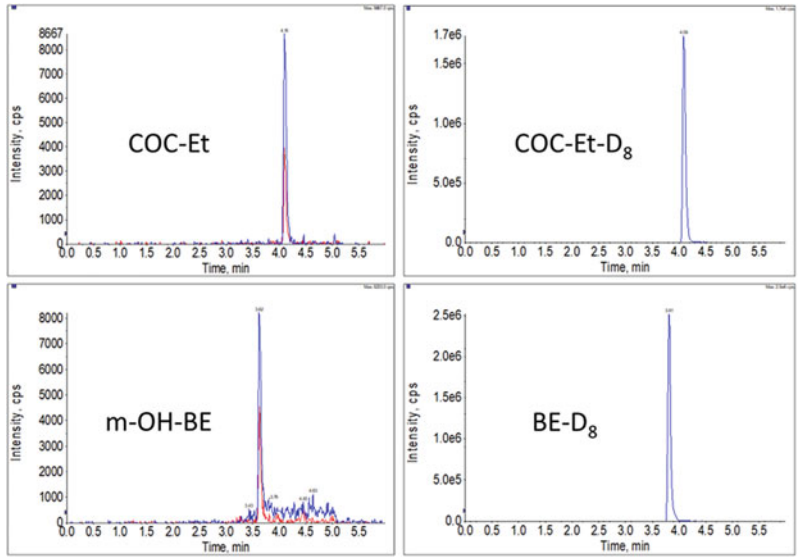

Fig. 2 (continued)

is evaluated during method validation. Guidelines on how to do this have been published elsewhere [24].

2. The blood should be kept for a maximum of about 5 days before hemolysis reaches a critical level. Hemolysis may affect spreading of the blood during DBS formation and extraction; therefore, hemolyzed blood may not be used. Freezing likewise induces hemolysis and is therefore not a suitable means of preservation of blood intended to produce DBS.
3. Preparation using volumetric glassware is not strictly necessary, as long as calibrators, QCs, and unknown samples are prepared with the same batch of extraction solvent. The optimal composition of the extraction solvent needs to be determined during method development. Generally, extraction solvents with an aqueous content between 0% and 20% produce relatively clean extracts that can usually just be diluted or evaporated and reconstituted before injection into the LC-MS/MS system. Extraction with a higher water content will also extract hemoglobin (and other proteins) from the DBS, and these extracts therefore require further cleanup before injection. In the presented method, an entirely aqueous extraction solvent was used and protein precipitation with acetonitrile was used as a further purification step to remove hemoglobin from the sample. Liquid-liquid extraction (LLE) or solid-phase extraction (SPE) are common alternatives for sample purification depending on the analyte.
4. When analyte stability in the sample matrix is assessed during method validation, these experiments should be carried out using EDTA blood in order to avoid interference from a

stabilizing effect of the NaF/K_2Ox. Additionally, incurred sample stability (without anticoagulant) can also be assessed.

5. For optimal transfer of the full volume, the pipette should best be held at a ca. 20° angle. Calibrators and controls should ideally be prepared freshly on the day of analysis. If storage of DBS is necessary, they should be kept at −20 °C or colder.
6. While commercially available card formats are more practical for the collection of authentic specimens, it is more economical to use large sheets (A4 or similar) of sample collection paper for the preparation of calibration and QC samples. Circular patterns can also be printed onto these sheets with a regular laser printer to aid with the positioning of the blood spots. Dashed lines should be used for the circles to minimize the interference of wax contained in the printer toner with the spreading of the blood on the paper.
7. The drying time may vary according to climate conditions. Long-term storage of DBS samples should always occur in sealable plastic bags with desiccant packages.
8. Drying racks are commercially available for card formats. For drying larger sheets of specimen collection paper, the authors use a DIY solution in which the paper is horizontally stretched in the air by attaching binder or foldback clips. The clips can be freely positioned and attached to the corners of the paper sheet.
9. Punchers of different diameters are commercially available, and the appropriate punch size needs to be evaluated during method development. Larger punches lead to increased sensitivity but may lead to problems in samples where patients were only able to generate small DBS. For 6 mm punches, a regular (European) office puncher available from stationary stores can be used.
10. Keeping the MeCN ice-cold (−20 °C) instead of at room temperature and combining it with a sufficient centrifugation time and speed (20 min, 14,000 × *g*) ensure removal of hemoglobin from the sample. Use of MeCN at room temperature and shorter centrifugation led to extracts that were still colored.
11. When analyzing volatile compounds such as amphetamines or cathinones, the addition of 20–40 μL of 0.25% hydrochloric acid (HCl) in MeOH can help to prevent analyte loss through evaporation by forming nonvolatile hydrochloride salts.
12. The composition of the reconstitution solvent should ideally reflect the chromatographic starting conditions and should have similar or lower elution strength than the starting conditions of the LC gradient to ensure optimal peak shape. Lowering the reconstitution volume can provide significant benefits

for method sensitivity. However, it should be large enough to ensure complete dissolution of the residue. Likewise, larger injection volumes may result in improved sensitivity.

13. Solvent blank samples (MeOH or mobile phase) should be run after the highest calibrator and highest QC, in order to assess potential carry-over, and periodically about every six samples. Carry-over should be less than 20% of the signal of the calibrator at the lower limit of quantitation.
14. When analyzing BE and nor-COC together, it is important to choose chromatographic conditions that allow full separation of the two compounds as they are isomers and share certain MRM transitions. Transitions that are specific for each analyte should be used for quantification. If only determination of BE is desired, interference from nor-COC may not be a significant problem in practice as nor-COC is only a minor metabolite occurring at low concentrations in authentic samples. Therefore, its potential for significant interference with the determination of BE is limited. However, it should be noted that nor-COC concentrations can be higher in users with a cholinesterase deficiency [25] or when EtOH is co-consumed [26].
15. Commonly, one quantifier transition (usually the most sensitive one) and one qualifier transition are chosen. In the case of isomeric or isobaric analytes, it is prudent to monitor additional qualifier transitions as is the case here for BE and nor-COC. The ratio of the different transitions, referred to as ion ratio, compared to an analytical standard measured on the same day (e.g., a calibration sample) should be used as an additional identification criterion. For LC-MS/MS applications, ion ratios of unknown samples may not differ more than ±30% from the ion ratios measured in standard samples [27].
16. The MS parameters presented here are instrument-specific and may need to be re-optimized when the method is transferred to another instrument.

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