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Loralie J. Langman
Christine L. H. Snozek *Editors*

LC-MS in Drug Analysis

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Preface

This book is intended to provide detailed LC-MS/MS procedures for the analysis of several compounds of clinical significance. The first two chapters provide the reader with an overview of mass spectroscopy, its place in clinical practice, its application of MS to TDM and toxicology, and the merits of LC-MS(/MS) and new sample preparation techniques. The remaining chapters discuss different approaches to screening for drugs of abuse and for general unknowns, as well as targeted measurement of specific analytes or classes of analytes including abused drugs, toxic compounds, and therapeutic agents.

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

Mayo Clinic, MN, USA

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Chapter 1

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

Catherine Hammett-Stabler 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. Though different in their application, each discipline depends upon accurate identification and quantification if the measurements are to be useful. Thousands of methods are described for drug analysis but until recently most have relied upon analytical tools, such as spectrophotometry and immunoassay, that suffer from lack of specificity and sensitivity. The introduction of methods based on mass spectrometry (MS), coupled to gas or liquid chromatography, has revolutionized these areas. The methods are proving to be robust, versatile, and economical. This chapter introduces 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.

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 immunoassay. It must be acknowledged that the methods utilizing each of these allowed TDM and toxicology to grow and mature but a new era is dawning as mass spectrometry promises to take the analysis of drugs and drug metabolites into new directions.

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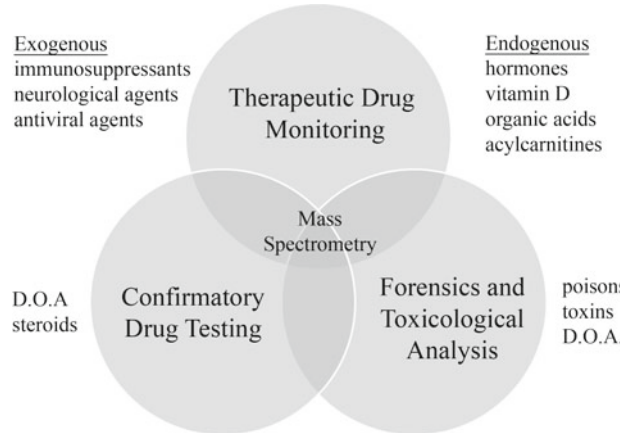

Fig. 1. Current applications of mass spectrometry in the clinical laboratory.

In recent years mass spectrometry coupled with liquid (LC-MS(/MS)) or gas (GC-MS) chromatography has emerged as a powerful tool for clinical and toxicology laboratories. Improvements in user interfaces, computing power, and column characteristics have expanded the potential of mass spectrometry-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 provides the reader with a number of applications to both disciplines which are introduced in the following pages. This chapter also discusses some of the issues one should consider when migrating to mass spectrometry-based methods.

2. Therapeutic Drug Monitoring

TDM 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 so were, in the 1980s, 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, the therapeutic targets for digoxin and tacrolimus are much lower than they were just a few years ago as recognition of toxicity has improved and protocols have changed. Clinical laboratories have thus replaced many of the TDM analyses using immunoassay with methods using LC-MS/MS (3–12).

Monitoring of immunosuppressant therapy for solid organ transplant patients using LC-MS/MS was perhaps one of the first areas in which the technique took hold. 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. Methodologies are now migrating from measurement of individual compounds separately to simultaneous quantification of immunosuppressant drugs from one whole blood sample using a single LC-MS/MS run (13–15). Whole blood samples are lysed and precipitated (either offline or online) followed by mass analysis with internal standards. Koster et al. compared LC-MS/MS with immunoassays for four immunosuppressants and reported that cyclosporine A and everolimus concentrations were 17 and 30% lower, respectively, compared to immunoassay (14), with the difference being attributed to the continued issue of cross-reactivity of immunoassay antibodies with cyclosporine and everolimus metabolites. Current research is focusing on the use of dried blood spot samples instead of whole blood for analysis (16–21). This approach provides a facile method to evaluate pharmacokinetics for individual patients through area under the curve (AUC) studies compared with the cumbersome collection of multiple venous blood samples.

Multicomponent antiviral therapy, particularly for HIV, needs careful monitoring to limit the development of viral resistance and

minimize toxicity. Highly active antiviral therapy (HAART) uses a cocktail of nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, and integrase inhibitors. Variation in metabolism between patients can be monitored through LC-MS/MS to maintain efficacy of the therapy. Most published methods simultaneously measure multiple compounds within a single plasma sample (22–27). In addition to plasma samples, LC-MS/MS analysis can be applied to alternate samples such as spinal fluid, breast milk, and semen when necessary for monitoring the antiviral therapies (28–31).

The narrow therapeutic window of digoxin makes it an ideal candidate for measurement using mass spectrometry (31–36). Furthermore, use of the technique overcomes the well-documented specificity and sensitivity issues of digoxin immunoassays. Mass spectrometry-based methods eliminate the cross-reactivity of digoxin-like interfering substance (DLIS) and also the issues with the digoxin overdose antidote, DigiBind. As noted previously, a lower therapeutic range is now recommended as increased toxicity and complications were noted (37, 38). Biological matrices include whole blood, serum, and plasma.

The aforementioned drugs or classes represent but a few of those for which LC-MS/MS methods have been described. Methods for antiepileptics, antidepressants, antimicrobials, 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. The fact that in these cases the toxic compound is unknown has created the need for recognition of signature product ion spectra that, when compared to an established library of relevant compounds, allows for unambiguous analyte identification. This systematic toxicological analysis (STA) or multi-analyte searching creates unique challenges for both clinical and forensic laboratories. Reference libraries of chemical spectra must be generated in-house or purchased. Screens encompassing >700 common abused, prescription, and over-the-counter medications using LC-MS/MS have now been described (6, 39–41). In contrast to the methods used in TDM in which specific drugs are quantified through the use of calibrations, these broad screening methods generally cannot be used to quantify exact concentrations of drugs that are present and are used for identification purposes when the compounds are present above a defined reportable limit of detection (LOD).

For many years, GC-MS has been considered the gold standard for confirmation of the presence of abused drugs in urine, particularly those sought in workplace and athletic drug testing. As methods and libraries have developed, many laboratories have turned to LC-MS/MS for these analyses. In the instance of pain management, confirmatory drug testing using LC-MS/MS allows 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 such as oxycodone. 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 (42).

The world of athletic drug testing is complicated by the ever-growing list of prohibited substances that are monitored for sporting events. Many of the agents elicit toxicological actions and their measurement becomes part of medical evaluations. The agents monitored include illicit as well as prescription drugs: Anabolic androgenic steroids, selective androgen receptor modulators, peptide hormones, growth factors, β-2 agonists, aromatase inhibitors, selective estrogen receptor modulators, diuretics, stimulants, narcotics, cannabinoids, alcohol, and glucocorticosteroids (43). Urine is the primary sample of testing but blood is used for hormones and growth factors.

In the forensic arena identification of drugs and their metabolites from samples related to criminal investigations provides challenges for the laboratory undertaking these analyses. Matrix effects from less than ideal samples may behave unusually compared to samples collected in the clinical setting. The myriad of potential toxins, poisons, and pharmaceuticals that could be present

in a single sample is daunting. Protocols with streamlined scan times and focused monitored ions have been optimized to cover as much chemical space as possible (9, 39, 44–46). Validation of the selectivity of the method should be tested to ensure proper identification. For example, Allen et al. reported a false positive for tramadol with samples containing venlafaxine during a urine LC-MS/MS screen for illicit drugs due to a shared transition ion (47).

Recent applications of mass spectrometry in forensics have been reported in postmortem identification of fentanyl in six overdoses cases by Peer et al. using direct injection of urine samples (48). Additionally, the designer hallucinogens 2,5-dimethoxy-4-iodophenethylamine (2C-I) and methylenedioxyamphetamine (MDA) were identified using LC-MS/MS in a urine sample from a patient who suffered a hemorrhagic stroke after presenting with hypertension, vasoconstriction, and decreased mental status (49).

4. Evaluation of Methods

A detailed discussion of LC or GC is beyond the scope of this book, though some features are discussed in this section. Generally, these techniques are used to separate the drugs of interest from other compounds present in the sample. Afterwards 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

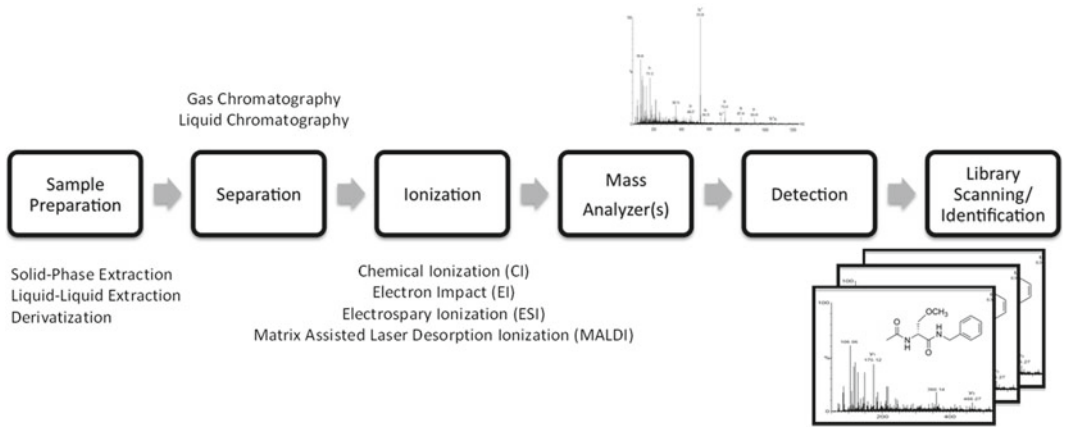

Fig. 2. Pipeline of steps involved in mass spectrometry analyses.

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), centrifuged, and the supernatant 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. If GC is to be used for separation, compounds with low volatility are derivatized allowing for separation in the gas phase prior to ionization.

Whether using LC or GC, it is usually necessary to separate the analytes of interest from each other and from unrelated compounds by the use of a column. Column chemistry and construction have improved greatly during the past 10 years, so much so that analyses that were considered restricted to one separation technique, i.e., LC versus GC, are now found using either. 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. Temperature programming, that is the precise, stepwise increase in the column temperature, is the equivalent of this in GC. 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.

After separation, the compounds are ionized prior to mass analysis. The most common techniques for ionization include chemical ionization (CI), electron impact (EI), electron spray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI). 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 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.

5. Quality Assurance

Each laboratory should develop a quality assurance program based upon its 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 LOD, limit of quantification (LOQ), linearity, selectivity, accuracy, precision, carryover, and matrix effects. For those drugs and analytes regulated under the Clinical Laboratory Improvement Act (CLIA) Amendments of 1988, the laboratory should use the mandatory precision limits to targeted day-to-day precision necessary for successful proficiency testing (50). Alternatively, one should consider the application of the analysis when setting precision and accuracy goals. If TDM is the application, the actual precision and accuracy needs may exceed those of CLIA. Digoxin serves as an excellent example again. Because of its narrow therapeutic index, an assay must be able to provide the same result within an equally narrow range on any given day. The CLIA limit for proficiency testing is

Table 1
Useful resources and documents for quality assurance programs

CLSI C43-A2	Gas chromatograph-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

20%, so the acceptable day-to-day precision is <7%. For most other drugs with TDM application, the CLIA limit is 25%.

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. 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 non-analytical 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 (50). 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).

A recent review of the state of forensic laboratories in the United States highlighted the lack of standardization and accreditation in some forensic laboratories (51, 52). Some of the outlined challenges regarding standardization and proficiency related to analyte identification will most likely be addressed through the development and adoption of standards of drugs and drug metabolites suitable for mass spectrometry. This paradigm shift has already taken place at the international level with the Scientific Working Group for the analysis of seized drugs (SWGDRUG), the International Laboratory Accreditation Program (ILAC), and the United Nations Office of Drug and Crime recently publishing guidelines for drug testing that include mass spectrometry as the premier analytical method for unambiguous analyte identification (53–55).

6. Future Perspectives

Conventional methods of drug testing using GC-MS or LC-MS(/MS) will continue to play an increasingly important role in clinical and forensic laboratories in the future. As new therapies enter the clinic, methods will be developed that permit accurate quantification for TDM applications. For example, methodologies for monitoring

everolimus (Zortress) and lacosamide (Vimpat) were reported before the drugs received approval by the Food and Drug Administration. Clinical chemists and forensic toxicologists have responded to a recent trend in the consumption of research chemicals such as substituted phenylethylamines (2C-B, 2C-E, 2C-I), the related benzodifurans (2C-B-FLY and Bromo-dragonfly), and synthetic cannabinoid ligands such as JWH-018 through the development of mass spectrometry screening technologies that can identify these designer drugs (56–58).

Future clinical applications of mass spectrometry are exploring global profiling of analytes present in patient samples. Examples of global-analyte scanning of urine, plasma, serum, and amniotic fluid are becoming commonplace in the research settings. Ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) and high-resolution mass spectrometry (HRMS) have emerged as promising tools to fingerprint metabolomes for clinical labs. Initial efforts have focused on the development of reproducible, robust protocols for metabolic profiling of samples, particularly for urine (59–63). A limited number of studies have attempted to discriminate between healthy and diseased samples using metabolic fingerprinting (64–66).

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 the quality of the data generated using mass spectrometry remains high.

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Chapter 2

LC-MS vs. GC-MS, Online Extraction Systems, Advantages of Technology for Drug Screening Assays

Pierre Marquet

Abstract

This chapter reviews recent applications of mass spectrometry to systematic toxicological analysis (STA), where extended lists of compounds of toxicological interest are screened, as well as to the general unknown screening (GUS), where all exogenous compounds present in a sample are tentatively detected and identified, without any preselection. Many recent improvements in sample preparation, chromatographic separation, gas chromatography-mass spectrometry, and above all liquid chromatography-mass spectrometry techniques are described, which are applicable or have been applied to STA and/or GUS, generally with promising results. These improvements come from miniaturization and automation of solid-phase extraction, turbulent-flow or ultrahigh-pressure liquid chromatography, linear ion traps, accurate (e.g., time of flight or orbital trap) mass spectrometry, as well as software refinements to alternate between different ionization modes or automatically interpret the results. It also shows that robust LC-MS/MS techniques already exist for STA or GUS, which are at least as efficient as the traditional techniques used in most toxicology laboratories, such as GC-MS or high-performance liquid chromatography with diode-array detection, as shown by three comparative studies. However, the major drawback of LC-MS/MS in the full-scan mode for STA or GUS is that it still lacks universal reference libraries due to insufficient reproducibility of LC-MS(/MS) mass spectra obtained with different instrument types.

Key words: Systematic toxicological analysis, General unknown screening, LC-MS, GC-MS, Mass spectral libraries

1. Introduction

The identification of drugs and toxic compounds, often at low levels, is an important goal in clinical and forensic toxicology, doping control analysis, and environmental analysis, where the compounds involved are often unknown. Gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography with diode-array detection (HPLC-DAD), and liquid chromatography-mass spectrometry (LC-MS) are the tools most often used in toxicology

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laboratories for identification, or confirmation of identity, of xenobiotics and their metabolites.

In all these fields, numerous methods have been developed for the analysis of particular target compounds, classes of compounds (e.g., therapeutic drugs, drugs of abuse, pesticides, environmental contaminants, and metabolites thereof), or for a more comprehensive screening of xenobiotics and their metabolites in biological samples. In fact, the screening and identification of compounds of interest before quantification is part of daily routine work (1, 2). Drug screening is a term that encompasses all the techniques allowing the detection in one run of a large range of compounds of pharmacological or toxicological interest in urine, plasma, serum, whole blood, and other body fluids, as well as hair or postmortem tissues or organs (3).

Many targeted screening methods involving single-stage mass spectrometry in the single ion monitoring (SIM) mode or tandem mass spectrometry in the selected reaction monitoring (SRM) mode have been developed for virtually all classes of drugs and toxic compounds. In addition to the selective and, if correctly applied (4), specific detection of the compounds targeted, they allow for their quantification.

The general unknown screening (GUS) of drugs and toxic compounds involves untargeted analytical techniques. Its aim is to detect as many compounds as possible in a matrix and tentatively identify them, either by comparison with libraries of mass spectra or by direct interpretation of an individual spectrum. Systematic toxicological analysis (STA) occupies an intermediate position between targeted and untargeted analysis. A limited (although sometimes very large) list of target compounds is screened for and, for those tentatively detected, rich and/or accurate mass spectral information is obtained, ensuring their specific identification.

This chapter focuses on recent STA and GUS procedures involving mass spectrometry and discusses the respective merits of GC-MS, LC-MS(/MS), and new sample preparation techniques.

2. GC-MS for STA and GUS: Recent Improvements

GC-MS has been the technique most employed for the GUS of compounds of toxicological relevance for the last three decades, owing to its universal fragmentation conditions and to the availability of huge mass spectral databases. In addition to being at the forefront of the development of GC-MS in clinical toxicology, in particular regarding GUS, the group of Maurer recently tested the freeware deconvolution software AMDIS (Automated Mass Spectral Deconvolution and Identification System) with their GC-MS GUS technique in urine (5). For this, after optimization of the AMDIS deconvolution and identification settings, they

compared the results obtained from 111 urine samples by manual and AMDIS data interpretation. They concluded that AMDIS gave results comparable or even superior to manual evaluation by an experienced toxicologist, but that it could only identify targets present in the user-defined MS library. As AMDIS-readable libraries have to be generated by users by converting commercial or personal libraries, this may narrow the range of toxicologically relevant compounds identified and is a current limitation of this promising tool.

Steiner and Larson (6) employed Direct Analysis in Real Time (DART), a new atmospheric pressure ionization technique that can be used for the analysis of solids, liquids, and gases with little or no sample preparation, merely by placing the test material into a heated gas flowing through the sampling area. Ionization in the positive mode is obtained by charging a heated helium gas stream, which subsequently reacts with the molecules on the surface of the sample to induce ionization. In this study, DART was coupled with a time-of-flight (TOF) MS analyzer operating at different collision-induced dissociation (CID) voltages, without prior chromatographic separation. This technique was able to detect many more compounds than GC-MS in 553 forensic case specimens; however, the authors emphasized that data obtained need to be examined very carefully as the spectra produced from multicomponent mixtures can become extremely difficult to interpret, interferences can result in falsely positive results, and differences in in-source CID spectra can arise for mixtures of compounds with widely varying proton affinities.

3. Recent LC-MS (/MS) Techniques for STA and GUS

Over the last 15 years or so, methods based on the use of HPLC coupled with single-stage or tandem MS detection have been reported for GUS and STA, fostered first by the necessity of detecting compounds not amenable to GC (i.e., highly polar, high-molecular-weight, or thermally labile compounds). It rapidly turned out that this coupling could detect a very large range of xenobiotics.

3.1. Single-Stage Quadrupole Mass Spectrometry

For single-stage MS, in-source CID at different energies was used to generate fragments and obtain rich enough spectra to be searched against libraries of spectra generated by the injection of reference materials in the same conditions. These methods, reviewed in detail elsewhere (7–9), have now been superseded by newer approaches.

3.2. Tandem Quadrupole Mass Spectrometry

Many LC-MS/MS methods have been published for the targeted analysis of a wide variety of drugs, mainly using SRM on triple quadrupole instruments. For instance, Gergov et al. (10) developed

a method for the screening of 238 drugs in blood using one SRM transition per compound and a compound-dependent collision energy (20, 35, or 50 eV), for a total cycle time of 6 s. However, the use of only one, or even two, SRM transition per compound is generally insufficient, yielding a significant number of false-positive findings (4). It should be kept in mind that MS in the SIM or SRM modes can never reach the identification power of a full mass spectrum (1).

Improvement with respect to these SRM methods was rendered possible by the availability of data-dependent acquisition or information-dependent acquisition (IDA), by which a tandem mass spectrometer can automatically switch from a "survey" mode to a "dependent" (or confirmation), full-spectrum MS/MS mode. In addition, the introduction of linear ion-trap-triple quadrupole (LIT-QqQ) hybrid instruments further extended the possibilities of LC-MS/MS in STA or GUS. In this instrument, the second mass analyzer can be used as either a conventional quadrupole mass analyzer or a linear ion trap, which by accumulation of ions provides enhanced full-spectrum sensitivity compared to a conventional quadrupole. The group of Weinmann used targeted SRM with up to 700 transitions as the survey detection mode, and the "enhanced" product ion (EPI) spectrum mode as the dependent mode (11). Whereas this procedure seems to be a more specific approach to STA as it allows searching rich spectra against those entered in libraries, the use of SRM as the survey mode cannot answer the more general clinical question as to whether an individual has been intoxicated at all, rather than intoxicated with a compound from a predefined list (12). Also, the use of only the positive-ion mode narrows the detection window.

Alternatively, the single-quadrupole, enhanced full-spectrum (EMS) mode has been used as the survey detection mode, with alternated polarities (13, 14). The major three ions in each Q3 MS were selected in the next three acquisitions and fragmented in the collision cell at three collision energies for each one, taking advantage of the accumulation capacity of the linear trap. Separate libraries were generated for the positive-ion and negative-ion modes by injecting pure solutions of drugs and toxic compounds, as well as by entering the MS/MS spectra of metabolites found in human samples, or even specifically produced by means of in vitro metabolic experiments (13). More than 1,000 MS/MS spectra in the positive mode and 250 in the negative mode were entered in the respective libraries, together with compound name, developed chemical structure, CAS number, retention time, relative retention time, and ultraviolet spectrum. A program was developed to automatically report the results of peak finding and library searching. Compounds not found by other screening or target techniques could be identified unambiguously by this LC-LIT-QqQ GUS technique in clinical toxicology cases (15). This technique is described in Chapter 11.

Libraries of mass spectra obtained through CID in the collision cell of triple quadrupole instruments have been developed for STA (16) or GUS (15). The robustness of CID mass spectra between instruments from the same or from different manufacturers, and thus the interchangeability of these libraries, has been investigated by different groups (17–22). These studies generally showed that the CID spectra were robust across laboratories equipped with the same instruments, or with instruments of the same brand, but that the relative intensity, and sometimes the nature of the fragments, differed across different instrument brands. However, in a recent study, product-ion spectra were generated at ten different collision energy values using a quadrupole-time-of-flight (Q-TOF) tandem mass spectrometer, filtered and entered in an MS/MS library. This library was further used to search unknown spectra generated on Q-TOF, QqQ, hybrid LIT-QqQ, and linear ion-trap-FTICR (Fourier transform ion cyclotron resonance) instruments in three different laboratories. By means of a sophisticated matching algorithm, the correct compound was retrieved as the best hit in 98.1% of cases and as the second best in the remaining 1.9% of cases (22).

Although also possible, the interpretation of unreferenced MS/MS spectra is a challenge because of the limited understanding of the fragmentation and rearrangement reactions involved and the limited number of fragments sometimes observed. As is seen below, even accurate-mass determination using high-resolution TOF or orbitrap mass spectrometers may not be sufficient to successfully identify unknowns.

3.3. Single-Stage Linear Ion-Trap Instruments

Mass spectral libraries dedicated to ion-trap instruments, whether three-dimensional or two-dimensional (i.e., quadrupole ion traps), have also been set up (23, 24), taking advantage of the easier-to-optimize CID conditions in ion traps due to the possibility of normalizing collision energies, and the more reproducible spectra obtained. Dulaurent et al. developed a GUS procedure for 320 pesticides and metabolites in blood using a linear ion-trap instrument in the positive and negative ions, MS^2 and then MS^3 modes (24). They generated MS^2 and MS^3 libraries of 450 and 430 spectra, respectively. Library searching was performed on MS^2 spectra and retention time, and positive results confirmed by manually checking the corresponding MS^3 spectrum. The limitations of this technique were that not all pesticides investigated could be detected and that the cycle time was quite long when continuously switching from the positive to the negative ionization modes. The authors admitted that, if necessary, it was possible to decrease the detection limits of some compounds by 10–100-fold by scanning MS^2 in only one polarity, owing to a shorter total scan time.

3.4. High-Resolution Mass Spectrometry

Liquid chromatography coupled to high-resolution TOFMS instruments, enabling accurate-mass determination, has also been employed for STA or GUS (25, 26). Identification has been based on the accurate mass, isotopic pattern, and retention time (27–29) of sample components, from which the atomic formula is calculated and searched against a database of relevant compounds, preferably using dedicated software (27). Alternatively, forward searching of compounds of toxicological interest in the full-scan TOFMS data was proposed by Ojanpera's group. This approach has been largely applied in the last couple of years in anti-doping laboratories (30–32). For instance, a generic LC-TOFMS method was developed and validated for 241 substances prohibited by the World Anti-Doping Agency, belonging to various categories (31). Positive identification was based on retention time and accurate mass, as compared to reference materials or compounds contained in urine samples from excretion studies. Limit of detection, extraction recovery, matrix effect, and repeatability were checked and the method successfully applied to the retrospective screening of a single designer drug, 4-methyl-2-hexanamine, in stored doping control samples.

When reference standards are not available, structures and thus elemental formulae of compounds of toxicological interest and their known or putative metabolites may be taken from the literature or inferred from expected metabolic pathways (33) and added to the database. However, as there are generally several compounds with the same elemental formula and molecular mass, and as their metabolites may also have the same masses, confirmation procedures may be necessary (1). Polettini et al. actually showed that no compound could be unambiguously identified in postmortem samples when searched against a library of 55,000 compounds of toxicological relevance (34). Lee et al. tried to overcome this limitation by using in-source CID to obtain more structural information and by building a mass spectral library using this approach (29). Alternatively to library searching, Pelander et al. relied on the prediction of fragmentation patterns using dedicated software (33). However, more application data will be necessary to demonstrate the reliability of compound identification without reference standards (2).

A next step in the development of LC-MS approaches in STA or GUS has been the use of two-stage, Q-TOFMS instruments able to generate accurate mass data of the parent as well as fragment ions directly attributable to the parent (21).

Only a few applications of orbital-trap (orbitrap) high-resolution mass spectrometers have been reported for STA or GUS so far. For the detection of 29 doping agents, an LTQ-orbitrap mass spectrometer equipped with an APCI ion source was used with in-source CID and acquisition in the positive ionization scan mode from 100 to 500 Da (35). The mass resolution of 60,000 full width at half maximum (FWHM) ensured a precision better than 2 ppm (using external calibration), while the limit of detection was better

than 100 pg/mL for all compounds. The possible fragmentation pathways of each agent were inferred from the fragments generated, using proprietor software. Despite the high selectivity of this technique, the authors admitted that some of the analytes were isomeric and had to be separated chromatographically. Using a different version of orbitrap, with no linear ion-trap upfront, Thomas et al. (36) developed a method without precursor ion selection, where spectra were acquired in the positive and negative modes in three alternated conditions: without fragmentation in the 100–1,000 Da range with a resolving power of 50,000 FWHM and then with CID at collision energies of 20 and 50 eV in the m/z 70–600 range with a resolving power of 25,000 FWHM. The resulting cycle time was <2 s. Compound identification was based on the accurate masses of the parent and fragment ions, sometimes both in the positive and negative ionization modes, as well as on their retention time. The authors validated their method for 32 doping agents, including some designer drugs recently introduced in the WADA lists for which no analytical technique was available at the time. Like the previous group, they emphasized the fact that this kind of method provides mass spectra containing all the desired information to identify unknown substances retrospectively.

A comparative study between TOFMS and orbitrap accurate mass spectrometry coupled with ultra-performance liquid chromatography (UPLC) was conducted in the field of hormone and veterinary drug residue analysis (37). Extracts from blank bovine hair were fortified with 14 steroid esters. All 14 compounds could be detected and their accurate mass measured at low ng/g concentrations using orbitrap mass spectrometry at a resolving power of 60,000. UPLC-orbitrap at a resolving power of 7,500 and UPLC-TOFMS at mass resolving power of 10,000 both failed to detect all steroid esters, owing to the inability to resolve analyte ions from co-eluting isobaric matrix compounds. High resolution can thus partly compensate for low signal-to-background noise concentration ratios, but the authors concluded that nonselective sample preparation is expected to aggravate the issue of false negative results obtained due to insufficient mass resolving power.

4. Ultrahigh Pressure (or Ultra-Performance) Liquid Chromatography

Quite a few of the recent, abovementioned STA or GUS techniques actually employed UPLC or UHPLC (29, 30, 36, 37) upfront mass spectrometry. However, the enhancement in chromatographic resolution produces very narrow (commonly 1–3 s wide) chromatographic peaks (38), which is only compatible with mass spectrometry cycle times at least threefold shorter (provided no polarity switching or alternated collision energies are used).

High-resolution mass spectrometers such as TOFMS or orbitrap instruments are more suited than QqQ instruments to acquire full-scan MS data within this time frame. High chromatographic resolution may thus be considered as a hindrance to rich MS data acquisition.

5. Comparison of GC-MS and LC-MS/MS Techniques for Screening Compounds of Toxicological Interest

Lee et al. compared their UPLC-TOFMS technique with HPLC-UV (REMEDiHS), in-house HPLC-DAD, full-scan GC-MS, and UPLC-MS/MS in the SRM mode for the analysis of 30 authentic urine samples (29). UPLC-TOFMS was able to detect 95 compounds, the REMEDiHS 47, GC-MS (without derivatization) 23, HPLC-DAD 14 (in a library of 594 UV spectra), and UPLC-MS/MS 23 (out of 170 targeted compounds). 94.7% of the compounds detected by TOFMS were confirmed by at least one of the other techniques, while the remaining four results could not be confirmed as false positive as the corresponding compounds were not included in the other techniques. On the other hand, three false negative results were noted. Although the "gold standard" comprised a combination of suboptimal techniques, these results advocate for the sensitivity and specificity of UPLC-TOFMS for GUS.

Lynch et al. compared five methods for GUS/STA (which they called comprehensive drug screening, or CDS) for their ability to detect drugs in 48 patient urine samples: LC-UV (REMEDi), full-scan GC-MS after acetylation of the extracts, full-scan LC-MS with in-source CID, LC-LIT-QqQ in the SRM information-dependent acquisition-enhanced product ion scan (SRM-IDA-EPI) mode (264 SRM transitions in the survey mode), and LC-LIT in the polarity switching, targeted MS^2 mode (39). They found that the LC-LIT and LC-LIT-QqQ methods identified 15% more drugs than the single-stage MS or LC-UV methods. However, none was able to detect all compounds and automatic library searching and reporting algorithms resulted in false positive and false negative results, which could be easily identified upon manual review of the raw data. The most common cause of false positive results was carryover, specially for LC-LIT, followed by nonspecific matching of spectra with <3 ions (in particular for LC-LIT-QqQ). It is worth noting that LC-LIT-QqQ led to tenfold more false negative results than LC-LIT (49.3% vs. 4.8%), which may also partly be attributed to the limited number of targeted SRM transitions with the former.

Another comparative study was conducted between GC-MS and an STA procedure developed on an LIT-QqQ instrument, following 100 drugs in the SRM survey mode (40). Ninety-five postmortem blood samples were analyzed in parallel resulting in

the detection of >400 drugs, and the two techniques yielded a surprising 98% concordance between them, despite 2 years of refrigerated storage between the two sessions of analyses.

One limitation of these three comparative studies is that the sample preparation procedures were different for all the analytical techniques compared, with or without urine hydrolysis, using liquid–liquid extraction (LLE), solid-phase extraction (SPE), or dilute and shoot, which does not actually allow for rigorous comparison of the respective merits of the hyphenated techniques. However, all three showed that LC-MS(/MS) was at least as efficient as the traditional techniques used for GUS/STA in most toxicology laboratories.

6. Extraction Strategies

Sample preparation and limits of detection are also important determinants of the efficiency of such methods. In particular, nonselective extraction procedures are necessary for good recovery of molecules in a wide polarity range, including highly polar drugs not amenable to GC-MS.

Filtration and injection or protein precipitation with acetonitrile and injection of the supernatant (the so-called dilute and shoot strategy) can provide a direct means to introduce samples into HPLC (41). However, the lack of a concentration step may limit the detection of some of the most potent drugs, while the absence of a purification step may favor matrix effects, hence false negative results.

Among the molecules targeted by LC-MS(/MS) GUS procedures are those not amenable to GC-MS, i.e., polar, acidic, thermally labile, or hydrophilic, and the extraction procedure should be chosen accordingly. Two LLE procedures can be used in parallel, one for acidic and one for basic compounds. SPE is also widely employed, either based on classical, mildly hydrophobic C8 or C18 mixed-mode phases, or on mixed-mode sorbents that can probably cope with compounds in the largest polarity range.

Though hardly addressed in the literature, emphasis should also probably be put on the very last step of sample preparation, i.e., the nature of the solvent used to reconstitute dry extracts, as the solubility of polar compounds can be poor in pure organic solvents.

Another recent possibility is to use online sample preparation techniques. Standard SPE cartridges can be used with commercial SPE automation coupled upfront with LC-MS/MS. Alternatively, microextraction by packed sorbent (MEPS) is a miniaturized SPE format intended to work with sample volumes as small as 10 µL. The MEPS sorbent bed is integrated into a syringe that allows for manipulations of low-volume samples, either manually or in

combination with certain autosamplers or sample preparation robots. The low solvent volume used for the elution of the analytes can be injected directly into GC or LC systems, hence providing completely automated MEPS/LC-MS or MEPS/GC-MS systems (42). Turbulent flow chromatography (TFC) is a column-switching technique based on direct injection of biologic samples, without previous extraction. Its main characteristic is the use of a first column packed with large particles of a stationary phase material and a high mobile phase flow rate, the combination of which generates a particular chromatographic behavior called turbulent flow, which allows retention of the small molecules of interest and exclusion of large biomolecules. However, drug–protein bonds have to be broken prior to injection into the system, generally using a first step of manual protein precipitation; otherwise the drug bound fraction would be eliminated with the proteins. To our knowledge, no published STA or GUS technique has employed online SPE, MEPS, or TFC so far. Although probably not superior to classic extraction techniques in terms of recovery yields and method sensitivity, these online techniques offer the advantage of automation. Actually, whatever the sample preparation procedure, one of the main problems when using LC-API-MS, particularly for GUS, is to detect even small signals against a high background noise. The critical point indeed is the signal-to-noise ratio (S/N), mainly determined by the purity of the extracts injected and the recovery of the compounds of interest.

7. Conclusion

Untargeted screening for unknown compounds by LC-MS is highly challenging. As a general rule, MS/MS in toxicology brings higher specificity and selectivity (higher S/N), as well as more structural information when an unknown chromatographic peak has to be explored. However, the first step for GUS is to detect unexpected compounds, which is not compatible with the classical SRM mode, either used alone or as the survey scan prior to a confirmatory, daughter ion scan mode. The major drawback of LC-MS/MS in the full-scan mode for STA or GUS is the lack of reference libraries that can be used on different apparatus types due to insufficient reproducibility of LC-MS(/MS) mass spectra obtained with different instrument types.

Major improvements have recently come from the MS part of the coupling: linear ion traps offer increased S/N ratio and MS^3 capabilities, while high-resolution (TOF or orbitrap) mass spectrometers offer higher mass precision, which greatly facilitates identification of unknown compounds and apparently shows the best performance in comparative studies. The time is probably

close now for a universal GUS procedure based on LC-MS, similar to but with much better performance than full-scan GC-MS, provided standardization of basic MS conditions can be agreed upon by vendors of mass spectrometers in order to share large libraries of spectra.

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Chapter 3

LC-MS/MS Techniques for High-Volume Screening of Drugs of Abuse and Target Drug Quantitation in Urine/Blood Matrices

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Abstract

Liquid chromatography-tandem mass spectrometry, employing electrospray ionization (ESI), has been applied in the analysis of many drugs and drug metabolites. Sample preparation has been an important part of this technique when analyzing biological samples. Here we describe a high-volume urine screening technique for approximately 40 different drugs of abuse as well as methods for quantification of many other drugs in serum, plasma, and whole blood. These techniques can be used in many different settings from clinical and forensic toxicology examinations to pharmacokinetic studies. Sample preparation procedures range from simple "dilute and shoot" methods to more extensive solid-phase extraction techniques.

Key words: Drugs of abuse, Tandem mass spectrometry, LC-MS/MS, Toxicology, Chromatography, GC-MS, Screening

1. Introduction

Broad spectrum, high-throughput analysis of drugs using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) emerged as a widely accepted technique in the late 1990s and early 2000s (1). This included applications for broad-spectrum screening as well as quantification and confirmation of drug identity (2–4). Previous to high-throughput mass spectrometric techniques, immunoassay systems were predominantly used for qualitative screening for many different drugs or families of drugs.

Drug testing governing bodies such as the National Institute for Drug Abuse (NIDA) and the SAMHSA (Substance Abuse and Mental Health Service Administration) developed guidelines, which included recommended cutoff concentrations for each drug or family of drugs analyzed (5). The obvious advantage to immunoassay systems is their ability to accommodate high-throughput

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processing for samples with little or no need to perform sample cleanup or extraction of analyte from matrix. The obvious disadvantages to using immunoassay-based drug testing are that substantial and variable cross-reactivity can exist for each species within a class of drugs, and that the assays often have poor specificity (6). This lack of specificity requires using an ultimately more reliable method for confirmation of drug identity in case of significant consequence or for legal purposes. The method of choice for confirmatory analysis has traditionally been gas chromatography-mass spectrometry (GC-MS) (7). By developing and assessing identification acceptance criteria when using multiple stage mass spectrometry for detection and quantification, it is possible to obtain confirmatory data in these high-throughput testing configurations (8). Specific criteria must be incorporated into methodologies including monitoring multiple mass transitions, evaluating the ratios of their relative intensities, as well as using strict retention window guidelines (3).

A major consideration for LC-MS/MS is the problem of ion suppression or enhancement. A non-optimized (for ion suppression/enhancement) analytical method can lead to poor precision and accuracy in quantitative methods (9). This is an important issue, which must be addressed in method development, validation, and routine use. Alterations of ionization efficiencies are a direct result of matrix effects (presence of co-eluting species). Two common ways of assessing matrix effect are either by a post-extraction addition method or the post-column infusion method. Modifications to sample extraction and/or chromatographic separation may be required to create a successful and robust quantitative method (10).

High-throughput concerns have for the most part been eliminated with fast chromatography utilizing sub 2 μm particle size separation columns. Fast analysis of small molecules requires effective and efficient chromatography as well as decreased analytical cycle time in the mass spectrometer. Newer systems have accomplished both of these tasks well (11). Elimination of tedious sample preparation and/or the introduction of automated extraction have become common to handle high-volume testing. Both off-line and online solid-phase extraction with column switching and turbulent flow chromatography have been used to perform sample preparation for pharmaceutical compounds and their metabolites. Run times for parent drug and several metabolites can be as short as 1 min (12).

Drug detection, identification, and quantification techniques are used for a wide variety of reasons. LC-MS/MS is a powerful analytical tool in metabolic studies in drug discovery, as a method to detect drugs as emerging contaminants in the environment, and for the study of pharmacokinetics in humans and animals. The technique is used for evaluating drug–drug interactions involving cytochrome P450 enzymes and evaluating new chemical entities.

High-throughput screening is used for the detection of illicit drugs in drug treatment patients, toxicological examination of clinical samples, forensic testing, and for law enforcement purposes. It is also used in therapeutic drug monitoring and for the detection of veterinary drug residues in milk, honey, and food products.

2. Materials

Methanol and acetonitrile are HPLC grade. Steam distilled water is used for all reagent preparation. All reagents are stored at room temperature unless otherwise indicated.

2.1. Standards and Reagents for General Screen

1. Commercial stock internal standards: Morphine-D_3, amphetamine-D_5, methylenedioxymethamphetamine (MDMA)-D_5, benzoylecognine-D_8, 7-aminoclonazepam-D_4, meperidine-D_4, methadone-D_9, clonazepam-D_4, diazepam-D_5, and carboxytetrahydrocannabinol (THC-COOH)-D_9 all at a concentration of 100 µg/mL (Cerilliant, Round Rock, TX). Stored at –20°C.
2. Intermediate stock internal standards: All intermediate internal standards except morphine-D_3 are prepared by diluting the commercial stock to a concentration of 1 µg/mL in methanol. Morphine-D_3 is prepared at a concentration of 2 µg/mL. No intermediate stock internal standard is made for THC-COOH-D_9. Stored at 4°C.
3. Working internal standard: Prepared by diluting commercial (THC-COOH-D_9 only) and intermediate internal standard solutions to a final volume of 200 mL in 60:40 (v/v, %) water:methanol with 0.1% formic acid, according to the concentrations specified in Table 1.
4. Commercial stock standards: Solutions of the targeted drugs were obtained in varying concentrations. Opened and unopened stock standards are stored at –20°C. Targeted drugs and stock concentrations are shown in Table 2.
5. Intermediate and working standards: Prepared as shown in Table 2. Intermediate standards are prepared in 50:50 (v/v) methanol:H_2O and are stored at –20°C. Working standards are prepared in drug-free urine, and stored at 4°C.
6. 0.2 M ammonium acetate buffer pH 4.9: Dissolve 1.54 g ammonium acetate in 100 mL H_2O. Vortex to dissolve.
7. Beta-glucuronidase: Beta-glucuronidase from *helix pomatia* at 2,701,900 Fishman units per gram (Sigma Aldrich, Oakville, ON, Canada) (see Note 1). Dissolve 0.1 g of enzyme in 12 mL of 0.2 M ammonium acetate buffer. Vortex vigorously and mix for 30 min. Spin and pipette off supernatant for use. Store protected from light at 4°C.

Table 1
Preparation of internal standard solution for routine drugs of abuse screen

Internal standard	Concentration of intermediate stock standard (μg/mL)	Volume of intermediate stock standard (μL)	Concentration of working internal standard (ng/mL)
Morphine-D_3	2	4,000	40
Amphetamine-D_5	1	4,000	20
[a]MDMA-D_5	1	1,000	5
Benzoylecognine-D_8	1	1,000	5
7-Aminoclonazepam-D_4	1	2,000	10
Meperidine-D_4	1	1,000	10
Methadone-D_9	1	1,000	10
Clonazepam-D_4	1	4,000	20
Diazepam-D_5	1	1,000	5
[a]THC-COOH-D_9	100 (commercial stock)	400	200

[a]*MDMA* methylenedioxymethamphetamine, *THC-COOH* carboxytetrahydrocannabinol

2.2. Reagents for LSD Screen in Urine

1. Internal standard: LSD-D_3, 25 μg/mL commercial stock. Dilute to 10 ng/mL in methanol, e.g., 10 μL LDS-D_3 stock diluted to volume in a 25 mL volumetric flask.
2. Cutoff control: Bio-Rad control C3 (0.65 ng/mL LSD), or an in-house control prepared in drug-free urine to the same concentration.

2.3. LC-MS/MS Solutions

1. Mobile Phase A: Distilled H_2O with 0.1% formic acid.
2. Mobile Phase B: HPLC grade acetonitrile with 0.1% formic acid.
3. Seal Wash: 10:90 (v/v) acetonitrile:H_2O.
4. Wash 1: 5:95 (v/v) acetonitrile:H_2O with 0.1% formic acid.
5. Wash 2: 50:50 (v/v) acetonitrile:H_2O with 0.1% formic acid.

2.4. Supplies

1. Waters Acquity UPLC with micro-titer well-plate auto-sampler (Waters Corp., Milford, MA, USA) and Waters Premier XE triple quadrupole mass spectrometer (Waters Corp., MicroMass UK Limited).
2. Agilent Zorbax™ Eclipse XDB-C18 (4.6×50 mm, 1.8 μm) (Agilent, Mississauga, ON, Canada) with 0.2 μm stainless steel frit guard assembly (Waters Acquity UPLC™ part #205000303).

Table 2
Standard concentrations (stock, intermediate, and calibration solutions) and cutoff values for routine drugs of abuse screen

Drug	Stock concentration (mg/mL)	Intermediate concentration (μg/mL)	Cutoff value (ng/mL)	Calibration concentrations (ng/mL)
7-Aminoclonazepam	0.1	20	100	0, 200, 400, 600
7-Aminoflunitrazepam	0.1	20	100	0, 200, 400, 600
α-Hydroxyalprazolam	0.1	20	100	0, 200, 400, 600
Alprazolam	1.0	20	100	0, 200, 400, 600
Amphetamine	1.0	50	500	0, 500, 1,000, 1,500
Benzoylecognine	1.0	15	150	0, 200, 400, 600
Clonazepam	1.0	20	100	0, 200, 400, 600
Cocaine	1.0	15	150	0, 150, 300, 450
Codeine	1.0	30	100	0, 150, 300, 450
Desalkylflurazepam	0.1	20	100	0, 200, 400, 600
Diazepam	1.0	20	100	0, 200, 400, 600
Diphenhydramine	1.0	20	100	0, 200, 400, 600
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	1.0	10	100	0, 200, 400, 600
Fentanyl	1.0	2.5	25	0, 25, 50, 75
Flunitrazepam	1.0	20	100	0, 200, 400, 600
Flurazepam	1.0	20	100	0, 200, 400, 600
Hydrocodone	1.0	30	100	0, 150, 300, 450
Hydromorphone	1.0	30	100	0, 150, 300, 450
Ketamine	1.0	20	100	0, 200, 400, 600
Lorazepam	1.0	20	100	0, 200, 400, 600
Methylenedioxyamphetamine (MDA)	1.0	50	500	0, 500, 1,000, 1,500
Methylenedioxy-*N*-ethyl-amphetamine (MDEA)	1.0	50	500	0, 500, 1,000, 1,500
Methylenedioxy-methamphetamine (MDMA)	1.0	50	500	0, 500, 1,000, 1,500
Meperidine	1.0	20	100	0, 500, 1,000, 1,500
Methadone	1.0	10	100	0, 150, 300, 450
Methamphetamine	1.0	50	500	0, 500, 1,000, 1,500

(continued)

Table 2 (continued)

Drug	Stock concentration (mg/mL)	Intermediate concentration (μg/mL)	Cutoff value (ng/mL)	Calibration concentrations (ng/mL)
Methylphenidate	1.0	10	100	0, 150, 300, 450
Morphine	1.0	30	100	0, 150, 300, 450
Nordiazepam	1.0	20	100	0, 200, 400, 600
Norfentanyl	1.0	2.5	25	0, 25, 50, 75
Normeperidine	0.1	20	100	0, 200, 400, 600
Oxazepam	1.0	20	100	0, 200, 400, 600
Oxycodone	1.0	30	100	0, 150, 300, 450
Phencyclidine (PCP)	1.0	2.5	25	0, 25, 50, 75
Pseudoephedrine	0.66	50	500	0, 500, 1,000, 1,500
Ritalinic acid	0.88	50	500	0, 500, 1,000, 1,500
Temazepam	1.0	20	100	0, 200, 400, 600
Carboxytetrahydrocannabinol (THC-COOH)	0.1	2.5	25	0, 25, 50, 75
Triazolam	1.0	20	100	0, 200, 400, 600

3. ep *Motion* 5070 automated dispenser system (Eppendorf AG), with single- and multi-channel heads, plus appropriate tips (Eppendorf, Mississauga, ON, Canada).
4. Deep well V-bottom 96-well plates.
5. Certified drug-free urine.
6. Urine toxicology controls. Commercial quality control materials are available, or in-house controls can be prepared targeting concentrations near the cutoff and throughout the analytical range. At least one control should contain conjugated metabolites such as glucuronides to ensure adequate enzymatic hydrolysis.

3. Methods

All procedures are carried out at room temperature; allow all standards, reagents, and samples to equilibrate to room temperature before using. Universal precautions should be taken at all times, as samples are potentially infectious.

3.1. Sample Preparation

1. All urine samples are processed from plastic tubes (75 mm × 12 mm). Samples not received in these tubes must be transferred prior to analysis.
2. All sample tubes should have 3–4 mL of urine for automated pipetting. Lower volumes will need to be manually pipetted, and larger volumes will need to be removed to prevent contaminating the pipette head (see Note 2).
3. Samples are inspected for adulteration prior to pipetting, and suspicious samples are noted on the plate map (see Note 3).
4. After inspection of samples, prepare the hydrolysis plate by pipetting 20 μL of beta-glucuronidase into each well of a deep well plate which will contain a sample or control. The wells containing standards and the negative control are not hydrolyzed and are not included on this plate.
5. Transfer 200 μL of each hydrolysis control and patient sample to the hydrolysis plate containing the beta-glucuronidase.
6. Place the hydrolysis plate on a plate shaker and shake for approximately 30 s.
7. Place the hydrolysis plate in a 60°C circulating water bath for 60 min.
8. Prepare the second deep well plate (analysis plate) for transfer while the hydrolysis plate is incubating. Add 200 μL of the negative control and standards according to the plate map.
9. Transfer 20 μL of each sample and control on the hydrolysis plate to the corresponding well on the analysis plate.
10. Add 180 μL of internal standard to each well of the analysis plate.
11. Cover the analysis plate with aluminum foil and label with the batch number prior to LC-MS/MS analysis.

3.2. LC-MS/MS Analysis

1. The injection volume is 15 μL. Each plate contains standards and controls for quantification and validation of the samples within (see Note 4). Each sample run takes approximately 5 min; running a full 96-well plate takes approximately 8 h.
2. LC-MS/MS instrument parameters are shown in Table 3. Compound specific parameters are shown in Table 4. The acquisition file is divided into separate functions in which the number of SRM transitions monitored is minimized. This provides enhanced sensitivity since the mass spectrometer does not scan the SRM transitions for every compound during the entire run. One isotopically labeled compound is chosen as internal standard for every function.
3. After a run is complete, the samples are reviewed and quantitated. Each peak is manually reviewed by a technologist, and

Table 3
LC-MS/MS instrument parameters

	Settings
Source (ES+)	
Capillary (kV)	3.3
Cone voltage	Compound specific
Extractor (V)	5.00
Radio frequency lens (V)	0.5
Source temp. (°C)	120
Desolvation temp. (°C)	400
Cone gas flow (L/h)	100
Desolvation gas flow (L/h)	800
Analyzer	
Low mass Q1 resolution	15.00
High mass Q1 resolution	15.00
Ion energy 1	0.8
Entrance	–2
Collision energy	Compound specific
Exit	0.2
Low mass Q2 resolution	15.00
High mass Q2 resolution	15.00
Ion energy 2	0.8
Multiplier (V)	665
Collision cell gas flow (mL/min)	0.11

quantitation is done using Waters QuanLynx software (Waters Corporation, Mississauga, ON, Canada). Controls from each run are plotted on Levey–Jennings charts to track performance over time, and ensure the validity of each day's run (Fig. 1).

4. The results are imported into the laboratory information management system (LIMS) as a text file. The LIMS system is programmed with the cutoff values (Table 2) for each drug and LIMS converts the quantitative results to qualitative reports (positive/negative) (see Note 5). The quantitative data is stored in LIMS for future reference.

3.3. LSD Screen

Screening for LSD in urine samples can also be done using LC-MS/MS, but it is not included as part of the routine drug screen due to different sample preparation requirements. The expected level of LSD in urine and thus the cutoff value is significantly lower than that for the drugs included in the screen, so an extraction and concentration step is required for LSD as opposed to simple dilution in the screen method.

1. Samples, standards, and blank (drug-free urine) are processed together.
2. Transfer 500 μL of each sample, control, standard, and blank to a clean glass tube.

Table 4
LC-MS/MS compound-specific parameters for routine drugs of abuse screen

Drug	Retention time (min)	Transitions[a]	Cone (V)	Collision energy (V)	Dwell (s)
Function 1: 0.8–1.40 min					
Morphine	1.11	**286.2 > 164.9**, 286.2 > 181.0	45	36	0.1
Hydromorphone	1.21	**286.1 > 185.0**, 286.1 > 157.0	45	33	0.1
Morphine-D_3	1.10	289.2 > 164.9	45	36	0.1
Function 2: 1.40–1.90 min					
Amphetamine	1.68	**136.0 > 118.9**, 136.0 > 90.9	12	10	0.01
Amphetamine-D_5	1.68	141.0 > 124.0	12	10	0.01
Pseudoephedrine	1.50	**166.2 > 148**, 166.2 > 133.1	12	10	0.02
MDA	1.74	**180.0 > 163.0**, 180.0 > 134.9	18	15	0.01
Codeine	1.49	**300.2 > 165.0**, 300.2 > 199.0	35	33	0.02
Oxycodone	1.65	**316.3 > 241.2**, 316.3 > 256.1	30	25	0.01
Function 3: 1.65–2.10 min					
Methamphetamine	1.80	**150.1 > 119.0**, 150.1 > 90.9	15	12	0.01
MDMA	1.85	**194.1 > 163.0**, 194.1 > 134.9	20	16	0.01
MDMA-D_5	1.84	199.1 > 165.1	20	16	0.01
MDEA	2.01	**208.1 > 163.0**, 208.1 > 135.0	25	12	0.01
Ritalinic acid	1.98	**220.5 > 84.0**, 220.5 > 91.0	25	22	0.01
Norfentanyl	2.00	**233.0 > 84.3**, 233.0 > 105.0	25	18	0.01
Hydrocodone	1.75	**300.3 > 199.1**, 300.3 > 171.0	45	30	0.01
Function 4: 1.85–2.30 min					
Ketamine	1.99	**238.0 > 179.0**, 238.0 > 125.0	40	20	0.01
Benzoylecognine	2.08	**290.2 > 167.9**, 290.2 > 149.9	30	25	0.01
Benzoylecognine-D_8	2.08	298.2 > 171.0	30	25	0.01
Function 5: 1.85–2.30 min					
Methylphenidate	2.26	**234.1 > 83.8**, 234.1 > 174.1	25	22	0.02
Normeperidine	2.37	**234.2 > 160.0**, 234.2 > 188.1	30	22	0.02
7-Aminoclonazepam	2.16	**286.2 > 222.1**, 286.2 > 222.1	40	25	0.02
7-Aminoclonazepam-D_4	2.16	290.1 > 226.1	40	25	0.02
Cocaine	2.39	**304.2 > 182.0**, 304.2 > 198.0	30	25	0.02
Function 6: 2.20–2.90 min					
PCP	2.62	**244.2 > 158.9**, 244.2 > 85.9	20	14	0.005
Meperidine	2.40	**248.1 > 220.1**, 248.1 > 174.0	35	26	0.005
Meperidine-D_4	2.40	252.2 > 224.1	35	26	0.005
Diphenhydramine	2.71	**256.0 > 167.0**, 256.0 > 152.0	20	15	0.005
7-Aminoflunitrazepam	2.42	**284.2 > 226.2**, 284.2 > 240.1	45	28	0.005
Fentanyl	2.67	**337.1 > 187.8**, 337.1 > 105.1	40	26	0.005
Flurazepam	2.68	**388.2 > 315.1**, 388.2 > 288.1	33	22	0.005
Function 7: 2.75–3.50 min					
EDDP	2.88	**278.2 > 234.1**, 278.2 > 249.2	40	25	0.008
Oxazepam	3.31	**269.2 > 241.1**, 269.2 > 231.1	25	22	0.008
Methadone	3.00	**310.3 > 265.1**, 310.3 > 223.1	25	20	0.008
Methadone-D_9	3.00	319.4 > 268.2	25	20	0.008
α-Hydroxyalprazolam	3.12	**325.1 > 297.1**, 325.1 > 279.1	50	28	0.008

(continued)

Table 4
(continued)

Drug	Retention time (min)	Transitions[a]	Cone (V)	Collision energy (V)	Dwell (s)
Function 8: 3.20–3.60 min					
Alprazolam	3.34	**309.2 > 281.1**, 309.2 > 205.0	45	33	0.005
Clonazepam	3.44	**316.2 > 270.1**, 316.2 > 302.1	40	25	0.005
Clonazepam-D_4	3.44	320.2 > 274.1	40	25	0.005
Lorazepam	3.38	**322.2 > 276.1**, 322.2 > 304.1	40	30	0.005
Triazolam	3.40	**344.2 > 309.1**, 344.2 > 316.1	40	28	0.005
Function 9: 3.35–4.50 min					
Nordiazepam	3.49	**271.1 > 140.0**, 271.1 > 164.8	42	24	0.01
Diazepam	3.96	**285.1 > 154.0**, 285.1 > 193.0	42	30	0.01
Desalkylflurazepam	3.56	**289.1 > 139.9**, 289.1 > 226.1	42	28	0.01
Diazepam-D_5	3.96	290.1 > 154.0	42	30	0.01
Temazepam	3.68	**301.0 > 255.1**, 301.0 > 283.0	25	20	0.01
Flunitrazepam	3.64	**314.0 > 268.1**, 314.0 > 300.1	40	30	0.01
Function 10: 4.48–5.20 min					
THC-COOH	4.85	**345.3 > 327.2**, 345.3 > 299.2	40	18	0.12
THC-COOH-D_9	4.85	354.3 > 308.2	40	18	0.12

[a]Quantifier transition in bold

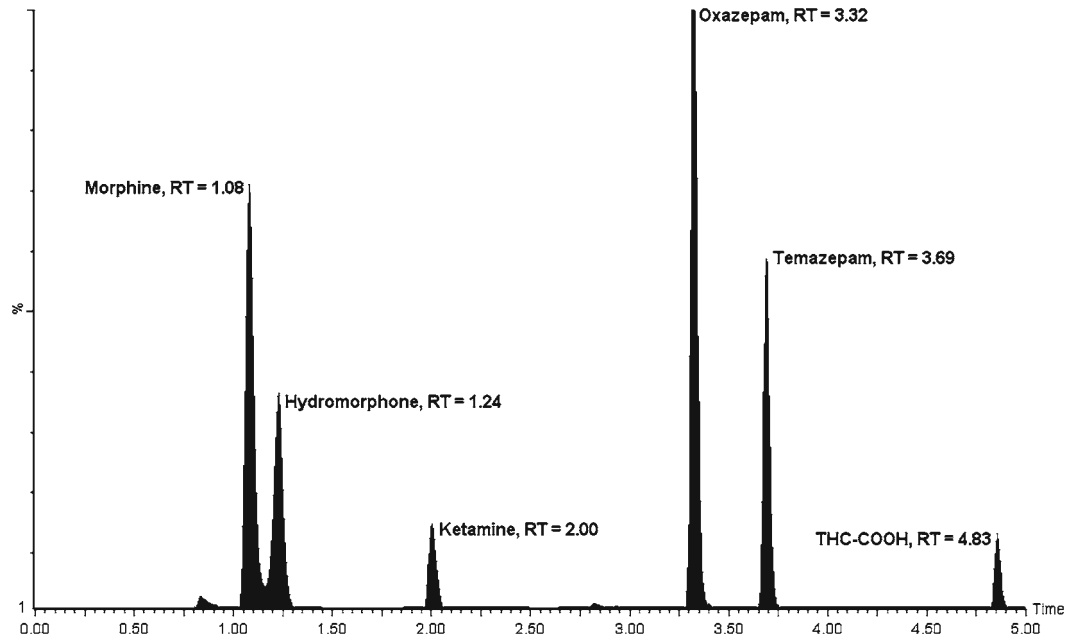

Fig. 1. Chromatogram of quantitation transitions from positive patient sample. Positive for morphine, hydromorphone, ketamine, oxazepam, temazepam, and THC-COOH.

Table 5
LC-MS/MS parameters for LSD screening

Drug	R.T.	Transitions[a]	Cone (V)	Collision energy (V)	Dwell (s)
LSD	2.35	**324.3 > 223.1**, 324.3 > 281.2	30	25	0.1
LSD-D_3	2.35	327.3 > 226.2	30	25	0.1

[a]Quantifier transition in bold

3. Add 50 μL of internal standard to each tube.
4. Add 200 μL of concentrated ammonium hydroxide and 2.0 mL of 1-chlorobutane to each tube. Mix on rocker for 30 min.
5. Centrifuge for 6 min at 2,200 × *g*.
6. Remove the top layer into a separate tube, and evaporate under nitrogen.
7. Reconstitute with 200 μL of 90:10 (v/v) acetonitrile (0.1% formic acid):H_2O (0.1% formic acid).
8. Transfer to a 96-well plate and run. Parameters for LSD analysis are shown in Table 5. LC-MS/MS parameters are the same as for the general screen (Table 3).

3.4. Quantitative Analysis

Routine analysis at our lab is a screen for common drugs of abuse, with results being reported as positive or negative for a specific analyte. In some cases, quantitative analysis is required, which requires additional steps (see Note 6). Some examples of the quantitative analyses performed are included below. In general, quantitative analysis can be performed for any analyte by increasing the number of calibration standards and accounting for the matrix by preparing the calibration standards in a similar matrix. Drugs and metabolites that have been quantitated in our laboratory using this methodology are detailed in Note 7. Many other drugs in the field of both clinical and forensic toxicology as well as therapeutic drug monitoring are suitable for this technique.

3.4.1. Opiate Quantitation in Whole Blood

1. Internal standard solution contains morphine-D_3, codeine-D_3, and hydromorphone-D_3 in methanol at the same levels as for routine screen. Opiate quantitation is typically done for morphine, hydromorphone, codeine, oxycodone, and hydrocodone.
2. Whole blood control material is used as the matrix for standards. Standards are prepared at 0, 150, 300, and 450 ng/mL.
3. 200 μL of sample is required for analysis. Add 2 × 200 μL aliquots of the internal standard solution to the whole blood

sample, vortexing the sample between additions in order to precipitate blood proteins. Centrifuge at 2,880 × *g* for 10 min.

4. Add 50 μL of the supernatant to the corresponding well of a 96-well plate, and dilute with 150 μL of 90:10 (v/v) acetonitrile (0.1% formic acid):water (0.1% formic acid).
5. In-house controls are prepared by spiking whole blood control material at two levels (100 and 400 ng/mL). These controls are prepared, aliquoted, and frozen.
6. Separate acquisition and quantitation methods are used for whole blood opiate quantitation to focus on the transitions of interest and optimize instrument scan time.

3.4.2. THC-COOH Quantitation in Urine

1. Samples are prepared in the same manner as for the routine drug screen. 20 μL of the hydrolyzed urine sample is added to 180 μL of working internal standard.
2. Standards are prepared in drug-free urine, at concentrations of 0, 25, 50, 75, and 100 ng/mL.
3. Standards and samples are placed in a 96-well plate for analysis. Separate acquisition and data analysis methods are used for THC-COOH quantitation, looking for only the transitions of interest.

4. Notes

1. Between 1 and 20 Fishman units of glucuronidase is needed per μL of urine to ensure complete hydrolysis (1). Supplied glucuronidase can vary in activity, but should be prepared so that this ratio can be maintained.
2. All specimens are stored at –20°C for 2 months after analysis. Samples of interest may be stored longer.
3. Adulterated samples that can be identified by sensory inspection prior to analysis can include dilute, colorless samples, unnatural colors (green, bright orange), or strong odors (bleach or cleaning agents). Suspected adulteration is noted on the report issued to the providers. These samples may be rejected or extracted to remove species that are potentially detrimental to the cleanliness of the mass spectrometer and thus to sensitivity of the instrument.
4. Standards and controls are run at the beginning, middle, and end of each plate to account for the continued hydrolysis that occurs in samples run later in the plate.
5. The LIMS system records the numeric concentration for each analyte as well as qualitative data, but only the positive/negative result is reported out to the provider.

6. Quantitative analysis is done routinely for opiates (commonly done for autopsy samples) and THC-COOH, while many other drugs are quantitated on a less frequent basis.
7. The following drugs and drug metabolites have been identified in a variety of biological matrices in our laboratory and quantitated using this methodology: 7-amino-clonazepam, 7-amino-flunitrazepam, alpha-hydroxy-alprazolam, alprazolam, amphetamine, benzoylecognine, clonazepam, cocaine, codeine, des-alkyl-flurazepam, diazepam, diphenhydramine, EDDP (methadone metabolite), fentanyl, flunitrazepam, flurazepam, hydrocodone, hydromorphone, ketamine, lorazepam, MDA, MDEA, MDMA, meperidine, methadone, methamphetamine, methylphenidate, morphine, nordiazepam, norfentanyl, normeperidine, oxazepam, oxycodone, PCP, pseudoephedrine, ritalinic acid, temazepam, THC-COOH, triazolam, nitrazepam, 7-a-nitrazepam, buprenorphine, norbuprenorphine, 6-MAM (heroin metabolite), LSD, enantiomeric methadone, enantiomeric EDDP, vincristine, nicotine, cotinine, cocaethylene (13, 14).

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Chapter 4

Benzodiazepines and Metabolites from Biological Fluids by Liquid Chromatography Electrospray Tandem Mass Spectrometry

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Abstract

Solid-phase extraction and liquid chromatography-tandem mass spectrometry are invaluable techniques for the determination of benzodiazepines and metabolites in biological matrices. The reason for using tandem mass spectrometry is to increase limits of detection without the need for chemical derivatization. Here we describe a technique for the detection of 26 benzodiazepines and metabolites at a detection limit of approximately 1–2 ng/mL in blood and 1–5 ng/mL in urine when screened using a data-dependent scan method.

Key words: Benzodiazepines, Solid-phase extraction, Liquid chromatography, Tandem mass spectrometry, Drug-facilitated sexual assault

1. Introduction

Benzodiazepines are one of the most commonly prescribed classes of drugs worldwide. They are frequently abused for their sedative and anxiolytic properties. The sedative and amnestic properties of benzodiazepines also render them desirable agents for use in a drug-facilitated sexual assault (DFSA) (1).

Although immunoassays and other screening techniques may be very sensitive for some benzodiazepines, they may not provide enough cross-reactivity for detecting the wide variety of benzodiazepines available today in the United States, and abroad (1). Laboratories that perform analysis of benzodiazepines for DFSA cases should validate their procedures to ensure that their method is capable of detecting benzodiazepines in accordance with the guidelines established by the Society of Forensic Toxicologists (SOFT) DFSA Committee (2). The benzodiazepine detection limits

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recommended by the SOFT DFSA Committee are as follows: 10 ng/mL for alprazolam, chlordiazepoxide, diazepam, hydroxyalprazolam, lorazepam, nordiazepam, oxazepam, and temazepam, and 5 ng/mL for 7-aminoclonazepam, 7-aminoflunitrazepam, clonazepam, flunitrazepam, hydroxytriazolam, and triazolam (2).

Biological specimens are qualitatively assayed and/or quantified for benzodiazepines and their metabolites (3). Specimens are mixed with deuterated internal standards. In order to maintain sensitivity without the need for derivatization, urine samples are hydrolyzed with H-2 helix pomatia beta-glucuronidase to cleave glucuronide conjugates. Samples are extracted using CLEAN SCREEN® DAU solid-phase extraction (SPE) cartridges. This extraction procedure is based on "Flunitrazepam, 7-Aminoflunitrazepam, and Desmethylflunitrazepam in Urine for GC/MS Confirmations Using 200 mg CLEAN SCREEN DAU Extraction Column" first published by United Chemical Technologies, Inc. (4). The published extraction is essentially followed intact. However, the extract is analyzed without derivatization by liquid chromatography electrospray tandem mass spectrometry. The use of data-dependent tandem mass spectrometry allows the mass spectrometer to scan for the most intense ion from a set list and to provide product ions for that precursor ion. It will then exclude that ion for a set period of time so that it can scan for the next intense ion to select and fragment. The assay presented here provides parameters for detection of 30 benzodiazepines, metabolites, and deuterated analogs.

2. Materials

2.1. Reagents and Equipment

Note that reagents are HPLC grade or greater. Hexane is pesticide grade.

1. CLEAN SCREEN DAU SPE cartridges: 200 mg/10 mL.
2. Beta-glucuronidase: Type H-2 from Helix pomatia, approximately 100,000 U/mL.
3. 0.1 M phosphate buffer, pH 6.0. To a 500-mL volumetric flask, add 400 mL of deionized water, 6.1 g of sodium phosphate monobasic monohydrate, and 1.6 g of sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify pH is 5.8–6.1. Bring to volume with deionized water. Store refrigerated in glass (see Note 1).
4. 1.1 M sodium acetate buffer. To a 100-mL volumetric flask, add 14.95 g of sodium acetate trihydrate, 60 mL of deionized water, and 2.2 mL of glacial acetic acid. Mix well to dissolve and bring to volume with deionized water. Verify pH is 5.0–6.0. Store refrigerated in glass.

5. SPE benzodiazepine wash solvent containing 20% acetonitrile/0.1 M phosphate buffer: Combine 80 mL of 0.1 M phosphate buffer (pH 6.0) with 20 mL of HPLC grade acetonitrile and mix well. Store in glass at room temperature.
6. Elution solvent: Ethyl acetate with 2% ammonium hydroxide. To a graduated cylinder, combine 98 mL of ethyl acetate and 2 mL of ammonium hydroxide. Make fresh daily.
7. LC Mobile Phase 60:40:0.03 methanol:water:ammonia: Combine 300 mL of HPLC grade methanol and 200 mL of HPLC grade water. Mix well and vacuum filter through a 0.5 μm PFTE membrane. Add 0.15 mL of concentrated ammonium hydroxide and mix gently. Verify pH >8.0. Store in glass at room temperature.
8. Liquid chromatography (LC)/ThermoFisher Scientific LTQ Ion Trap Mass Spectrometer or equivalent capable of tandem mass spectrometry and data-dependent scanning.
9. Alltech Alltima C18 (or equivalent) analytical column: 15 cm × 2.1 mm × 5 μm.

2.2. Standards and Controls

1. Methanolic Internal Standard Stocks: D_5-alprazolam, D_7-flunitrazepam, D_4-norflunitrazepam, and D_7-7-aminoflunitrazepam, all at 100 μg/mL (Cerilliant Corp, Round Rock, TX).
2. D_5-Alprazolam Internal Stock Standard Working Solution: 1 μg/mL. To a 100 mL volumetric flask add 1.0 mL of D_5-Alprazolam Stock Standard (100 μg/mL) and dilute to the mark with deionized water. Store refrigerated in glass.
3. Deuterated Flunitrazepam and Metabolites Internal Standard Working Solution: 0.5 μg/mL. To a 100 mL volumetric flask add 0.5 mL each of D_7-flunitrazepam, D_4-norflunitrazepam, and D_7-7-aminoflunitrazepam 100 μg/mL stock standards, and dilute to the mark with deionized water. Mix well. Store refrigerated in glass.
4. Benzodiazepine Multi-Component Mixture-8 Stock Standard: 250 μg/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam (Cerilliant Corp, Round Rock, TX).
5. Benzodiazepine Multi-Component Mixture-8 Working Solution: 5 μg/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam. To a 50 mL volumetric flask add 1.0 mL of Benzodiazepine Multi-Component Mixture-8 Stock Standard (250 μg/mL) and dilute to the mark with methanol. Store below 0°C in glass (see Note 2).
6. Negative control urine and blood, drug free.

7. Low positive control urine: 20 ng/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam. To a 100 mL volumetric flask, add 400 µL Benzodiazepine Multi-Component Mixture-8 Working Solution (5 µg/mL) and dilute to the mark with negative control urine. Solution is frozen in 5 mL aliquots (see Note 3).
8. High positive control urine: 100 ng/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam. To a 100 mL volumetric flask, add 2 mL Benzodiazepine Multi-Component Mixture-8 Working Solution (5 µg/mL) and dilute to the mark with negative control urine. Solution is frozen in 5 mL aliquots (see Note 3).
9. Positive control blood: 25 ng/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam. To a 50 mL volumetric flask, add 250 µL Benzodiazepine Multi-Component Mixture-8 Working Solution (5 µg/mL) and dilute to the mark with negative control urine. Solution is frozen in 2 mL aliquots (see Note 3).

3. Methods

3.1. Sample Preparation for Blood Specimens

1. Into properly labeled test tubes (16 × 100 mm or 16 × 150 mm) add 2 mL of negative control, positive control, or questioned blood specimens (see Note 4).
2. Add 40 µL of the Deuterated Flunitrazepam and Metabolites Internal Standard Working Solution and/or 20 µL of the D_5-Alprazolam Internal Standard Working Solution to each specimen. This results in a final concentration of 10 ng/mL for each deuterated analog (see Note 5).
3. Add 7 mL of 0.1 M phosphate buffer. Vortex.
4. Centrifuge at high speed for at least 15 min.
5. Transfer supernatant liquid to a clean test tube, leaving any solids behind.
6. Bring volume up to 9 mL with deionized water.
7. Verify pH of specimen is in the range of 6 ± 0.5.

3.2. Sample Preparation for Urine Specimens

1. Into properly labeled test tubes (16 × 100 mm or 16 × 150 mm) add 5 mL of negative control, high and low positive controls, or questioned urine specimens (see Note 6).
2. Add 100 µL of the Deuterated Flunitrazepam and Metabolites Internal Standard Working Solution and/or 50 µL of the D_5-Alprazolam Internal Standard Working Solution to each specimen. This results in a final concentration of 10 ng/mL for each deuterated analog (see Note 7).

3. If required, enzymatically hydrolyze the sample as follows. Otherwise, proceed to step 4 (see Note 8).
   (a) Adjust pH to 5.2 ± 0.5 with 1 mL of 1.1 M sodium acetate buffer coupled with the addition of 30 µL of H-2 Helix pomatia beta-glucuronidase (approximately 100,000 U/mL).
   (b) Vortex, cap, and incubate for 4–6 h at 37°C (see Note 9).
   (c) Cool to room temperature.
4. If hydrolysis step is omitted, add 1 mL of deionized water to all tubes.
5. Add 3 mL of 0.1 M phosphate buffer. Vortex.
6. Centrifuge at high speed for at least 10 min.
7. Transfer supernatant liquid to a clean test tube, leaving any solids behind.
8. Verify pH of specimen is in the range of 6 ± 0.5.

3.3. Solid-Phase Extraction of Biological Samples (Blood and Urine)

1. Prerinse SPE extraction cartridge by adding 3 mL of methanol at 1–2 mL/min.
2. Condition column with 3 mL of deionized water followed by 2 mL of 0.1 M phosphate buffer.
3. Load sample on SPE cartridge at approximately 1–2 mL/min. Do not allow sorbent to dry.
4. Wash column with 2 mL of deionized water followed by 2 mL of wash solvent.
5. Dry column for 1 min.
6. Wash column with 2 mL hexane.
7. Dry column for 1 min.
8. Rinse column with 2 mL of deionized water.
9. Dry column for 1 min.
10. Elute with 2.5 mL of freshly prepared elution solvent at approximately 0.5 mL/min.
11. Evaporate eluent at ≤40°C. Do not overdry (see Note 10).
12. Reconstitute extracts with 20–100 µL of mobile phase or with 20–100 µL of 50:50 methanol:deionized water (see Note 11). Vortex.
13. Analyze 5–10 µL of the extracts by LC/MS (ESI) using the instrumental conditions that follow. MS analysis can be full scan, MS/MS, or data dependent (see Note 12, Figs. 1 and 2).

3.4. Instrumental Conditions

3.4.1. Liquid Chromatography Parameters

(a) Mobile Phase: 60:40:0.03 methanol:deionized water:ammonia.
(b) Flow rate of 0.2 mL/min, isocratic flow. Run time is 35 min.
(c) Oven temperature at 30°C.
(d) Column C-18, length of 15 cm, internal diameter of 2.1 mm, and a particle size of 5 µm.

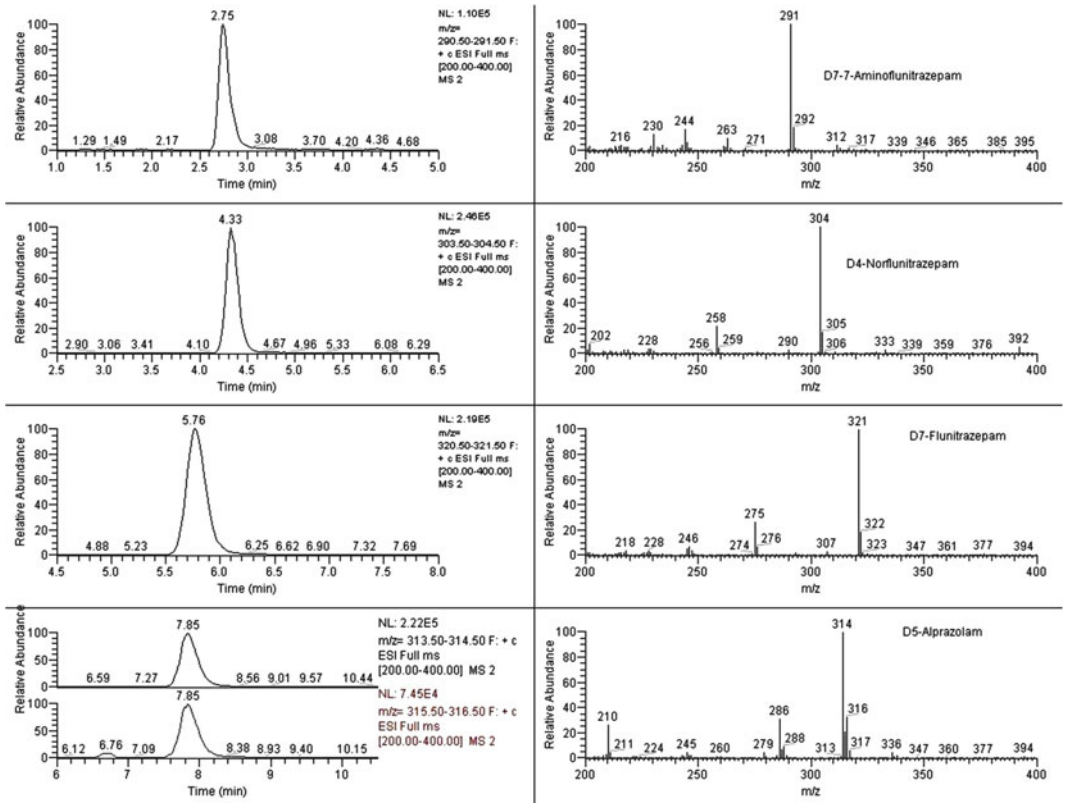

Fig. 1. RIC Benzodiazepine and Metabolite Internal Standards—full scan.

3.4.2. Mass Spectral Parameters

(a) ESI source settings: Sheath gas flow rate of 14, aux gas flow rate of 14, spray voltage 5 kV, capillary temperature 225°C, capillary voltage 47 V, and tube lens 215 V (see Note 13).

(b) Scan Event #1: Electrospray ionization mode, scan mode set at full scan, and scan range of 200–400 amu.

(c) Scan Event #2: Electrospray ionization mode, scan mode set at products ion MS/MS, precursor ion set at most intense from list 40% relative collision energy (see Note 14), 2 amu isolation width, software control product scan range, 500 counts threshold, enabled dynamic exclusion, 20 count, 30-s repeat duration; ±0.5 amu, 20-s exclusion duration.

4. Notes

1. Adjust phosphate buffer pH by addition of 0.1 M dibasic sodium phosphate (increases pH) or 0.1 M monobasic sodium phosphate (decreases pH), as necessary.

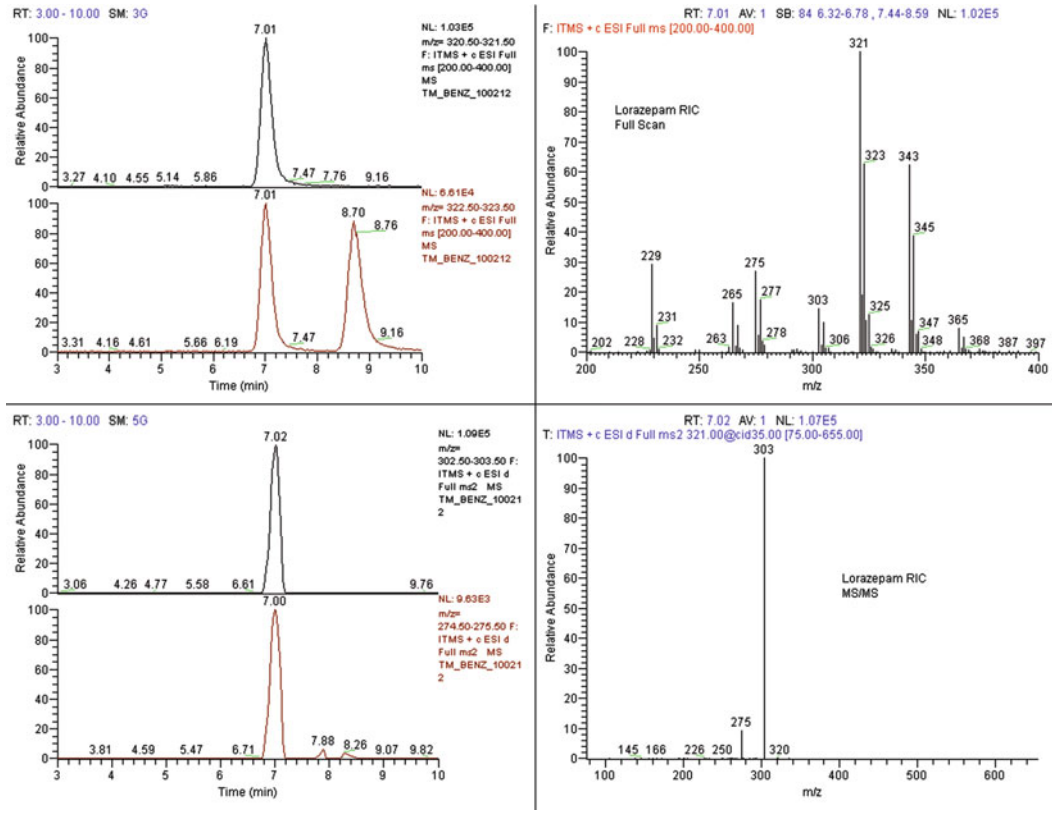

Fig. 2. RIC of lorazepam, full scan and MS/MS.

2. A 1:1 dilution of the 5 µg/mL Benzodiazepine Multi-Component Mixture-8 Working Solution with deionized water may be made and analyzed as a 2.5 µg/mL LC/MS performance standard. In order for the LC to be considered in good operating condition, molecular ion traces for each benzodiazepine in the performance standard should generate Gaussian-shaped chromatographic peaks. The retention times of the eight analytes should be within ±5% of the previous analysis of the performance standard. In order for the MS to be considered in good operating condition, the correct mass assignments for each of the eight analytes in the performance standard should be present.
3. Values for low and high positive control urine and positive control blood are suggested values and can be modified.
4. Analysis of smaller sample sizes has been done on a nonroutine basis. Validation should be conducted prior to analysis (see Table 1).

Table 1
Selected benzodiazepines and metabolites: limits of detection

Drug	Blood (ng/mL) (based on 2 mL sample size)	Urine (ng/mL) (based on 5 mL sample size)
Alprazolam	2	2
Hydroxyalprazolam	2	2
Diazepam	1	1
Nordiazepam	2	2
Flunitrazepam	1	1
Nitrazepam	1	1
Oxazepam	2	5
Clonazepam	1	1
Temazepam	1	2
Lorazepam	1	1

5. If specifically interested in lower amounts of analyte, blood specimens can be spiked with 20 μL of the Deuterated Flunitrazepam and Metabolites Internal Standard Working Solution and/or 10 μL of the D_5-Alprazolam Internal Standard Working Solution. This results in a final concentration of 5 ng/mL for each deuterated analog.
6. Analysis of smaller sample sizes has been performed on a non-routine basis (see Table 1).
7. If specifically interested in lower amounts of analyte, urine specimens can be spiked with 50 μL of the Deuterated Flunitrazepam and Metabolites Internal Standard Working Solution and/or 25 μL of the D_5-Alprazolam Internal Standard Working Solution. This results in a final concentration of 5 ng/mL for each deuterated analog.
8. Hydrolysis is strongly recommended when screening for low levels of highly conjugated benzodiazepines or metabolites.
9. Overnight hydrolysis is not recommended and does not improve recovery.
10. Overdrying eluent will lead to poor recovery of internal standards and analytes of interest. Take samples just to dryness.
11. If either a low detection limit is required or low specimen volume is used, reconstituting with less volume is recommended

to increase sensitivity. Reconstitution can be done with either mobile phase or methanol:water due to availability at the bench.

12. To justify the existence of a peak, its baseline signal to peak-to-peak ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least tenfold greater than that for any observed peak at similar retention time in a negative control or solvent blank injected just prior to the sample. The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of a reference standard, an extracted positive control, or an appropriate deuterated analog. The mass spectrum of the analyte of interest should match that of a reference standard or an extracted positive control within a reasonable degree of scientific certainty (the major product ion should be present at 100%; if the ion ratio in the known spectrum is >40% then the ion ratio in the unknown spectrum should be within 25% relative; if the ion ratio in the known spectrum is ≤40%, then the ion ratio in the unknown spectrum should be within 10% absolute).
13. ESI source setting flow rates on a Thermo Scientific LTQ XL are arbitrary units.
14. Any subset of the above target precursor ions may be selected for confirmation of a given analyte or group of analytes. Collision energy for a selected subset may also be adjusted based upon previously determined optimizations: typically range from 25 to 45% (see Table 2).

Acknowledgements

This extraction procedure is derived from "Flunitrazepam, 7-Aminoflunitrazepam, and Desmethyl-flunitrazepam in Urine for GC/MS Confirmations Using 200 mg CLEAN SCREEN DAU Extraction Column"; first published by United Chemical Technologies (UCT) Inc., UCT has granted approval for the reprinting of this extraction procedure.

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Table 2
Benzodiazepines and metabolites: LC-MS/MS information

Drug/metabolite	Relative retention time[a]	Precursor ion	Major fragment peaks	Optimum relative collision energy
Internal Standards				
D_7-7-aminoflunitrazepam	0.32	291	NA	N/A
D_4-norflunitrazepam	0.60	304	N/A	N/A
D_7-flunitrazepam	0.76	321	N/A	N/A
D_5-alprazolam	1.00	314	N/A	N/A
Drugs				
7-Aminoclonazepam	0.27	286	250, 222	40
7-Aminonitrazepam	0.29	252	121, 224	45
7-Aminoflunitrazepam	0.32	284	264, 256	45
n-Desmethylflunitrazepam	0.60	300	254	45
Bromazepam	0.64	316	288, 209	45
4-Hydroxynordiazepam	0.65	287	165, 259	45
Alpha-hydroxytriazolam	0.70	359	331, 341	40
Clonazepam	0.70	316	270, 288	45
Nitrazepam	0.73	282	236, 254	45
Flunitrazepam	0.76	314	268, 286	45
Alpha-hydroxyalprazolam	0.80	325	297, 279	40
Estazolam	0.84	295	267, 192	45
Triazolam	0.92	343	308, 315	45
Lorazepam	0.95	321	303, 275	25
Norchlordiazepoxide	1.00	286	269, 241	35
Alprazolam	1.00	309	281, 274	45
Oxazepam	1.01	287	269, 241	25
Desalkylflurazepam	1.07	289	261, 226	45
Temazepam	1.15	301	283, 255	25
Lormetazepam	1.20	335	317, 289	25
Alpha-hydroxymidazolam	1.24	342	324, 203	35
Chlordiazepoxide	1.36	300	283, 241	30
Nordiazepam	1.48	271	243, 140	45
Diazepam	1.76	285	257, 228	45
Midazolam	1.94	326	291, 244	45
Halazepam	2.71	353	325, 222	45

N/A not applicable
[a]Relative to D_5-alprazolam

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Chapter 5

Opiate Screening and Quantitation in Urine/Blood Matrices Using LC-MS/MS Techniques

Jeff C. Eichhorst, Michele L. Etter, Patricia L. Hall, and Denis C. Lehotay

Abstract

Here we describe a high-volume urinary screening technique for opiate drugs as well as other narcotic analgesics. We also describe methods for quantification of the same drug species in serum, plasma, and whole blood. Screening and quantitation of these types of drugs have presented many challenges, among them the potentially low levels in both abuse and therapeutic situations. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), employing electrospray ionization (ESI), has been able to provide the sensitivity needed for the analysis of many drugs and metabolites. These techniques can be used in many different settings from clinical and forensic toxicology examinations to pharmacokinetic studies and, with appropriate considerations, be applied to different sample matrices. Sample preparation procedures range from simple "dilute and shoot" methods to more extensive solid-phase extraction techniques.

Key words: Opiates, Opioids, Tandem mass spectrometry, LC-MS/MS, Toxicology, Chromatography, GC-MS, Screening

1. Introduction

Naturally occurring opiates such as morphine or codeine and semi-synthetic opiates such as hydrocodone or hydromorphone have been used as essential pain management components for decades. Since drugs such as these are used chronically in pain treatment and because they possess both euphoric and addictive qualities, they are commonly used illicitly as well. Other drugs, which produce morphine-like effects, are labeled as opioid drugs and are commonly used in similar situations.

Screening for opiates is routinely performed by immunoassay techniques, with confirmation by gas chromatography-mass spectrometry (GC-MS) (1). Drug testing governing bodies such as the National Institute for Drug Abuse (NIDA) and the SAMHSA

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(Substance Abuse and Mental Health Service Administration) developed guidelines which included recommended cutoff concentrations for each drug or family of drugs analyzed. The advantage to immunoassay systems is their ability to accommodate high-throughput processing for samples with little or no need to perform sample cleanup or extraction of analyte from matrix. The disadvantage to using immunoassay-based drug testing is the substantial and variable cross-reactivity that can exist for each species within a class of drugs resulting in decreased specificity.

By developing and assessing identification acceptance criteria when using multiple-stage mass spectrometry for detection and quantification, it is possible to obtain confirmatory data in these high-throughput testing configurations (2). Specific criteria must be incorporated into methodologies including monitoring multiple mass transitions, evaluating the ratios of their relative intensities, as well as using strict retention window guidelines (3).

Opiate analysis has been practiced using different methodologies for many years; however, interpretation of results is often complex due to the fact that metabolism to active metabolites creates species that are also available commercially and have abuse potential (4). As well urinary parent/metabolite ratios change over time and accumulations are affected by chronic dosing. Inclusion of some of the "nor" metabolites such as norcodeine, noroxycodone, and norhydrocodone may facilitate interpretation of which drug was administered (4).

The presence of urinary hydrocodone after chronic codeine use and the presence of urinary hydromorphone after chronic morphine use are also documented (5, 6). Another concern with interpretation of urinary opiate results involves the possibility that other species may be detected due to impurities generated from the manufacturing process (7). Analysis of samples using more sensitive techniques such as LC-MS/MS may further complicate the interpretation of results by detecting these impurities. Clinicians and laboratories testing urine specimens for these drugs and making predictions about patterns of use should be aware of this possibility.

A major consideration for any LC-MS/MS application is the problem of ion suppression or enhancement. A non-optimized (for ion suppression/enhancement) analytical method can lead to poor precision and accuracy in quantitative methods (8). This is an important issue, which must be addressed in method development, validation, and routine use. Alterations of ionization efficiencies are a direct result of matrix effects (presence of co-eluting species). Two common ways of assessing matrix effect are either by a post-extraction addition method or the post-column infusion method. Modifications to sample extraction and/or chromatographic separation may be required to create a successful and robust quantitative method (9).

High-throughput concerns have for the most part been eliminated with fast chromatography utilizing columns with particle sizes smaller than 2 μm. Fast analysis of small molecules requires effective and efficient chromatography as well as decreased analytical cycle time in the mass spectrometer. Newer systems have accomplished both of these tasks well (10). Elimination of tedious sample preparation and/or the introduction of automated extraction have become common to handle high-volume testing. Both off-line and online solid-phase extraction with column switching and turbulent flow chromatography have been used to perform sample preparation for pharmaceutical compounds and their metabolites (11). Run times for parent drug and several metabolites can be as short as 1 min (12).

2. Materials

Methanol and acetonitrile are HPLC grade. Steam distilled water is used for all reagent preparation. All reagents are stored at room temperature unless otherwise indicated.

2.1. Standards

1. Commercial stock internal standards: Morphine-D_3, codeine-D_3, hydromorphone-D_3, 6-acetylmorphine-D_3, oxycodone-D_3, hydrocodone-D_3, dihydrocodeine-D_3, oxymorphone-D_3, norhydrocodone-D_3, noroxycodone-D_3, all at a concentration of 100 μg/mL (Cerilliant, Round Rock, TX). Stored at −20°C.
2. Intermediate stock internal standards: To separate 10 mL volumetric flasks, add 100 μL of each commercial stock internal standard. Fill to volume with methanol and mix. Final concentration of each intermediate is 1 μg/mL. Store at −20°C.
3. Working internal standard for screen: To a 200 mL volumetric flask, add 4 mL of each intermediate internal standard solution as shown in Table 1. Fill to volume with 60:40 (v/v, %) H_2O:methanol with 0.1% formic acid. Final concentration of each internal standard is 20 ng/mL.
4. Working internal standard for quantitative blood assay: To a 200 mL volumetric flask, add 4 mL each of morphine-D_3, codeine-D_3, and hydromorphone-D_3. Dilute to volume with 100% methanol. Final concentration of each is 20 ng/mL.
5. Commercial stock standards: Solutions of the targeted drugs were obtained in varying concentrations (Cerilliant). Opened and unopened stock standards are stored at −20°C. Targeted drugs and stock concentrations are shown in Table 1.
6. Intermediate and working standards: Prepared as shown in Table 1. Intermediate standards are prepared in 50:50 (v/v, %) methanol:H_2O and are stored at −20°C. For the screen,

Table 1
Standard concentrations (stock, intermediate, and calibration solutions) and cutoff values for routine opiate screen in urine

Drug	Stock concentration (mg/mL)	Intermediate concentration (ng/mL)	Cutoff value (ng/mL)	Calibration concentrations (ng/mL)
6-Monoacetylmorphine	0.1	5,000	10	0, 10, 25, 50
Codeine	1.0	30,000	100	0, 150, 300, 450
Dihydrocodeine	1.0	30,000	100	0, 150, 300, 450
Hydrocodone	1.0	30,000	100	0, 150, 300, 450
Hydromorphone	1.0	30,000	100	0, 150, 300, 450
Morphine	1.0	30,000	100	0, 150, 300, 450
Norcodeine	1.0	30,000	100	0, 150, 300, 450
Noroxycodone	1.0	30,000	100	0, 150, 300, 450
Norhydrocodone	1.0	30,000	100	0, 150, 300, 450
Oxycodone	1.0	30,000	100	0, 150, 300, 450
Oxymorphone	1.0	30,000	100	0, 150, 300, 450

working standards are prepared in drug-free urine and stored at 4°C. For quantitation in serum/plasma or blood, working standards are prepared in drug-free whole blood. Aliquot and freeze at –20°C for up to 3 months.

7. Working controls for quantitative blood assay: Prepare as described for standards, by spiking drug-free whole blood to 100 and 400 ng/mL. Aliquot and freeze at –20°C.

2.2. Reagents

1. 0.2 M ammonium acetate buffer: Dissolve 1.54 g ammonium acetate in 100 mL H_2O. Vortex to dissolve.
2. Beta-glucuronidase: Beta-glucuronidase from *helix pomatia* at 2,701,900 Fishman units per gram (Sigma Aldrich, Oakville, ON, Canada) (see Note 1). Dissolve 0.1 g of enzyme in 12 mL of 0.2 M ammonium acetate buffer. Vortex vigorously and mix for 30 min. Spin and pipette off supernatant for use. Store protected from light at 4°C.

2.3. LC-MS/MS Solutions

1. Mobile Phase A: Distilled H_2O with 0.1% formic acid.
2. Mobile Phase B: HPLC grade acetonitrile with 0.1% formic acid.
3. Seal Wash: 10:90 (v/v, %) acetonitrile:H_2O.
4. Wash 1: 5:95 (v/v, %) acetonitrile:H_2O with 0.1% formic acid.
5. Wash 2: 50:50 (v/v, %) acetonitrile:H_2O with 0.1% formic acid.

2.4. Materials

1. Waters Acquity UPLC with micro-titer well-plate auto-sampler (Waters Corp., Milford, MA) and Waters Premier XE triple quadrupole mass spectrometer.
2. Agilent Zorbax™ Eclipse XDB-C18 (4.6×50 mm, 1.8 μm) (Agilent, Mississauga, ON, Canada) with 0.2 μm stainless steel frit guard assembly (Waters).
3. ep *Motion* 5070 automated dispenser system (Eppendorf AG), with single- and multi-channel heads, plus appropriate tips (Eppendorf, Mississauga, ON, Canada).
4. Urine toxicology controls C3 and C4 (Bio-Rad Laboratories, Montreal, QC, Canada).

3. Methods

All procedures are carried out at room temperature; allow all standards, reagents, and samples to equilibrate to room temperature before using. Universal precautions should be taken at all times, as samples are potentially infectious.

3.1. Sample Preparation (Urine Screening)

1. For automated pipetting, all urine samples are processed from plastic tubes (75 mm×12 mm). Samples in different tubes must be transferred prior to analysis.
2. All sample tubes should have 3–4 mL of urine for automated pipetting. Lower volumes will need to be manually pipetted, and larger volumes will need to be removed to prevent contaminating the pipette head (see Note 2).
3. Samples are inspected for adulteration prior to pipetting, and suspicious samples are noted on the plate map (see Note 3).
4. After inspection of samples, the hydrolysis plate is prepared by pipetting 20 μL of beta-glucuronidase into the wells of a deep well plate which will contain a sample or a control. The wells containing standards and the negative control are not hydrolyzed.
5. Following the plate map, and using the automated pipettor, 200 μL of each sample is transferred to the hydrolysis plate containing the beta-glucuronidase.
6. Place the hydrolysis plate on a plate shaker and shake for approximately 30 s.
7. Place the hydrolysis plate in a 60°C circulating water bath for 60 min.
8. Prepare the second deep well plate (analysis plate) for transfer while the hydrolysis plate is incubating. Add the negative control and standards according to the plate map.

Table 2
LC-MS/MS instrument parameters

	Settings
Source (ES+)	
Capillary (kV)	3.3
Cone voltage	Compound specific
Extractor (V)	5.00
Radio frequency lens (V)	0.5
Source temp. (°C)	120
Desolvation temp. (°C)	400
Cone gas flow (L/h)	100
Desolvation gas flow (L/h)	800
Analyzer	
Low mass Q1 resolution	15.00
High mass Q1 resolution	15.00
Ion energy 1	0.8
Entrance	–2
Collision energy	Compound specific
Exit	0.2
Low mass Q2 resolution	15.00
High mass Q2 resolution	15.00
Ion energy 2	0.8
Multiplier (V)	665
Collision cell gas flow (mL/min)	0.11

9. Transfer 20 µL of each sample and control on the hydrolysis plate to the corresponding well on the analysis plate using the automated pipettor.
10. Add 180 µL of internal standard to each well of the analysis plate using the automated pipettor.
11. Cover the analysis plate with aluminum foil and label with the batch number prior to LC-MS/MS analysis.

3.2. LC-MS/MS Analysis

1. The injection volume is 15 µL. Each plate contains standards and controls for quantification and validation of the samples within (see Note 4). Each sample run takes approximately 5 min; running a full 96-well plate takes approximately 8 h.
2. LC-MS/MS instrument parameters are shown in Table 2. Compound-specific parameters are shown in Table 3. The gradient used for chromatographic separation is shown in Table 4, and the flow rate through the column is 0.6 mL/min (see Note 5). The total run time for the method is 5.2 min. The acquisition file is divided into separate functions in which the number of SRM transitions monitored is minimized. This provides enhanced sensitivity since the mass spectrometer does not scan the SRM transitions for every compound during the entire run. One isotopically labeled compound is chosen as internal standard for every function (see Note 6).

Table 3
LC-MS/MS compound-specific parameters for routine opiate screen

Drug	R.T.	Transitions[a]	Cone (V)	Collision energy (V)	Dwell (s)
Function 1: 0.8–1.40 min					
Morphine	1.11	**286.2 > 164.9**, 286.2 > 181.0	45	36	0.1
Hydromorphone	1.21	**286.1 > 185.0**, 286.1 > 157.0	45	33	0.1
Morphine-D_3	1.10	289.2 > 164.9	45	36	0.1
Oxymorphone	1.14	**302.1 > 227.0**, 302.1 > 284.0	36	30	0.1
Function 2: 1.30–1.90 min					
Norcodeine	1.41	**286.0 > 164.8,** 286.0 > 120.9	45	40	0.05
Dihydrocodeine	1.43	**302.1 > 199.0**, 302.1 > 171.0	40	40	0.05
Codeine-D_3	1.48	303.2 > 165	35	33	0.02
Codeine	1.49	**300.2 > 165.0**, 300.2 > 199.0	35	33	0.02
Noroxycodone	1.62	**302 > 227,** 302 > 186.9	30	30	0.02
Oxycodone	1.65	**316.3 > 241.2**, 316.3 > 256.1	30	25	0.02
Function 3: 1.65–2.10 min					
6-Acetylmorphine	1.68	**328.2 > 211,** 328.2 > 193	45	30	0.02
Norhydrocodone	1.71	**286 > 199,** 286 > 241	40	28	0.02
Hydrocodone-D_3	1.74	303.1 > 199.1	45	30	0.01
Hydrocodone	1.75	**300.3 > 199.1**, 300.3 > 171.0	45	30	0.01

[a]Quantifier transition in bold

Table 4
Gradient elution parameters

Time (min)	%A	%B
0	90	10
1.2	75	25
4	10	90
4.1	2	98
4.7	90	10

Mobile Phase A: Distilled H_2O with 0.1% formic acid
Mobile Phase B: HPLC grade acetonitrile/0.1% formic acid

3. After a run is complete, the samples are reviewed and quantitated. Each peak is manually reviewed by a technologist, and quantitation is done using Waters QuanLynx software. Controls from each run are plotted on Levey–Jennings charts to track performance over time, and ensure the validity of each day's run.
4. The results are imported into the LIMS system as a text file. The LIMS system is programmed with the cutoff values (Table 1) for each drug and LIMS converts the quantitative results to qualitative reports (positive/negative) (see Note 7). The quantitative data is stored in LIMS for future reference.

3.3. Quantitative Analysis in Serum, Plasma, and Whole Blood

Routine analysis at our lab is a screen for common drugs of abuse on urine samples, with results being reported as positive or negative for a specific analyte, based on an established "cutoff" value. In many cases, quantitative analysis is required, which requires additional steps (see Note 8).

1. Add 200 µL of each standard, control (see Note 9), and unknown sample to a deep-well plate well.
2. Add 2 × 200 µL aliquots of the internal standard solution (see Note 10) to the whole blood sample, vortexing between additions in order to precipitate blood proteins. Centrifuge at 16,000 × *g* for 10 min.
3. Add 50 µL of the supernatant to the corresponding well of a 96-well plate, and dilute with 150 µL of 90:10 (v/v, %) acetonitrile (0.1% formic acid):H_2O (0.1% formic acid).
4. Separate acquisition and quantitation methods are used for whole blood opiate quantitation to focus on the transitions of interest and optimize instrument scan time (see Note 11).

4. Notes

1. Between 1 and 20 Fishman units of glucuronidase is needed per µL of urine to ensure complete hydrolysis (1). Supplied glucuronidase can vary in activity, but should be prepared so that this ratio can be maintained.
2. All specimens are stored at –20°C for 2 months after analysis. Samples of interest may be stored longer.
3. Adulterated samples that can be identified by sensory inspection prior to analysis can include dilute, colorless samples, unnatural colors (green, bright orange), or strong odors (bleach or cleaning agents). Suspected adulteration is noted on the report issued to the providers. These samples may be rejected or extracted to remove species that are potentially detrimental to the cleanliness of the mass spectrometer and thus to sensitivity of the instrument.
4. Standards and controls are run at the beginning, middle, and end of each plate to account for the continued hydrolysis that occurs in samples run later in the plate.
5. With the number of possible parent species as well as metabolites, specifically in samples from patients practicing multiple opiate use, isobaric interferences may pose a problem. In most jurisdictions there are no official criteria for compound identification using LC-MS/MS techniques for illicit drug detection or for clinical analysis (13). There are, on the other

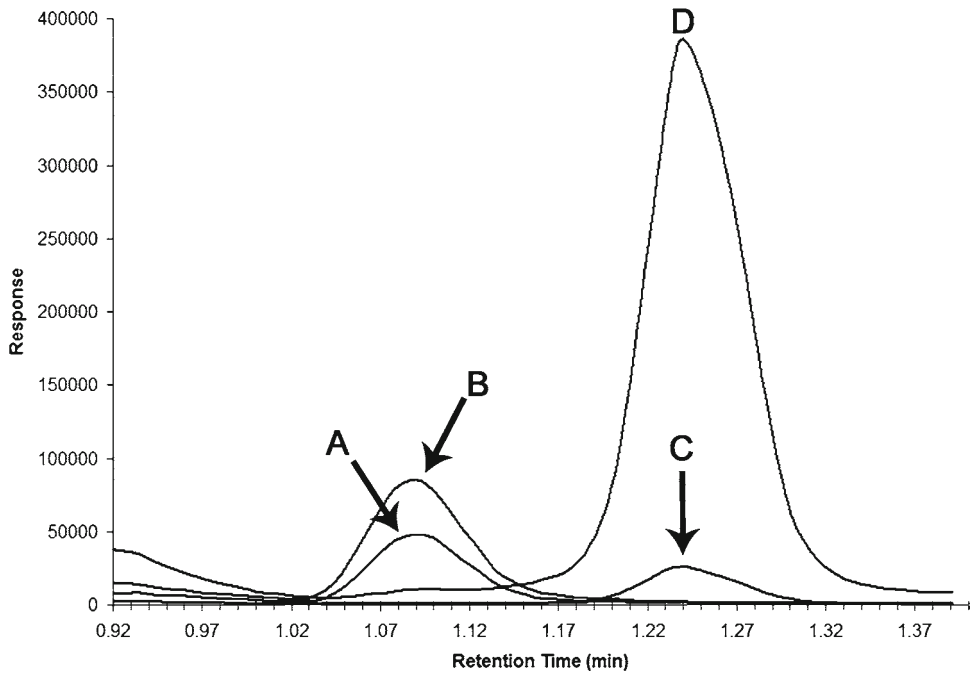

Fig. 1. Chromatographic resolution ($R = 1.5$) of morphine and hydromorphone when unique transition selection is not possible. (**a** and **b**) Morphine standard showing response to transitions used for morphine (286.2 > 164.9) and hydromorphone (286.1 > 185). (**c** and **d**) Hydromorphone standard showing response to transitions used for hydromorphone (286.1 > 185) and morphine (286.2 > 164.9).

hand, European Union rules in relation to pesticide residue detection as well as by the World Anti-doping Agency for detection of drugs in sport (13). Utilization of appropriate chromatography to provide chromatographic separation of isobaric species *must* be a part of method development for clinical applications of opiate detection and quantitation.

6. Our method employs small particle size chromatography (<2 μm), which allows improved chromatographic resolution and inherently narrower peak widths. Typically peak widths are around 0.1 min and the coefficient of variation for retention times consistently around 1% (14). Identification of each opiate drug is based on an accurate relative retention time, protonated molecule of a precursor ion, a minimum of two selective fragment ions, and relative ratios of these ions as compared to an authentic standard from the same run. Isotopically labeled standards are used for each compound in quantitative applications. Internal standards for screening purposes are limited to one or two per experiment for practical reasons and to make the interpretation less complicated.
7. The LIMS system records the numeric concentration for each analyte as well as qualitative data, but only the positive/negative result is reported out to the provider (see Fig. 1).

Table 5
Individual internal standards used for quantitation of specific opiate drugs in serum and whole blood

Drug	Internal standard	Internal Std MRM transition
Morphine	Morphine-D_3	286.1 > 164.9
Codeine	Codeine-D_3	303.2 > 165
Hydromorphone	Hydromorphone-D_3	289.1 > 185
6-Acetylmorphine	6-Acetylmorphine-D_3	331.2 > 165
Oxycodone	Oxycodone-D_3	319.3 > 244.2
Hydrocodone	Hydrocodone-D_3	303 > 199.1
Dihydrocodeine	Dihydrocodeine-D_3	305 > 199.1
Oxymorphone	Oxymorphone-D_3	305 > 230
Nor metabolite quantitation		
Norcodeine	Codeine-D_3	303.2 > 165
Norhydrocodone	Norhydrocodone-D_3	289.1 > 199.1
Noroxycodone	Noroxycodone-D_3	305 > 287

8. Quantitative analysis is done routinely for opiates (most often for autopsy samples). Our routine practice is to perform a screen for opiates in urine and whole blood precipitate. Based on the results of this initial screen, we then apply a quantitative analysis appropriate for the species observed in the screen. Clinical history is used in this process as well and prescription drug history is utilized in autopsy cases. In general, quantitative analysis can be performed for any analyte by increasing the number of calibration standards, using ion ratio criteria, establishing retention time criteria, and accounting for the matrix (whole blood, serum, or plasma). Matrix effects are most commonly overcome by using a well-matched isotopically labeled internal standard; however, special attention to this phenomenon during method development is essential (see Table 5 and Fig. 2).
9. Internal and external whole blood quality control samples (when available) are analyzed to ensure calibration accuracy. Autopsy specimens are spiked with standard solutions so that a recovery for each blood is acquired. This is performed due to the condition of some autopsy samples and their potential for "abnormal" matrix effect.
10. The working internal standard solution for opiate quantitation is used as a "crash" solution for precipitating blood proteins. Opiate quantitation is typically done for morphine,

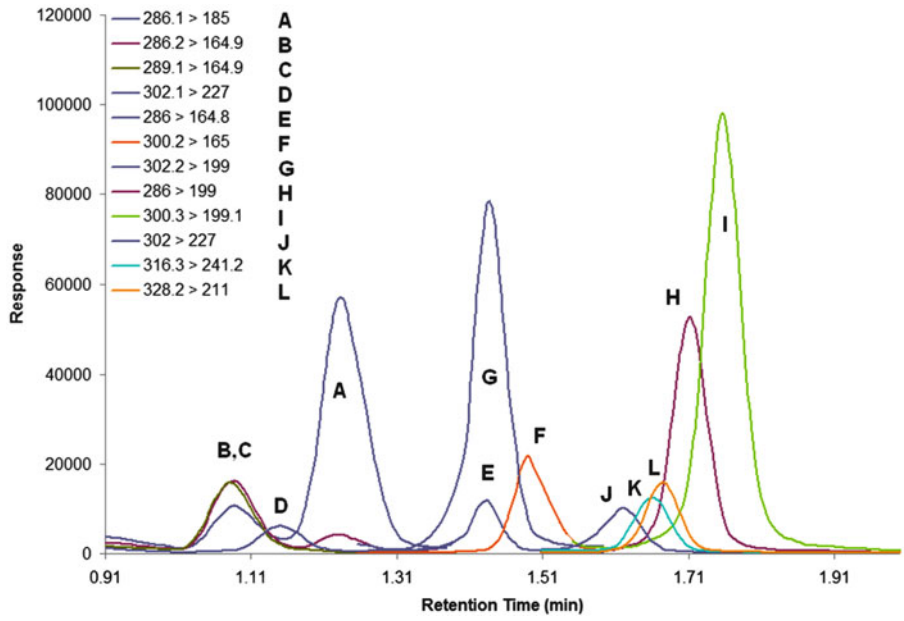

Fig. 2. Reconstructed chromatogram from all three experiments of MRM transitions for opiates and metabolites. For less complexity only one labeled internal standard is shown (Morphine D3). A unique transition–selection must be used for species, which are not chromatographically resolved (e.g., codeine and metabolites—*E* norcodeine standard. *F* Codeine standard. *G* Dihydrocodeine standard). *A* hydromorphone, *B* morphine, *C* morphine D3, *D* oxymorphone, *H* norhydrocodone, *I* hydrocodone, *J* noroxycodone, *K* oxycodone, *L* 6-MAM.

hydromorphone, codeine, oxycodone, and hydrocodone. When other opiates are quantitated, the internal standard is selected as shown in Table 5, and made up at the same level as for the routine screen. Only one labeled internal standard is required for each time function.

11. Compromise between chromatographic separation and overall run time is decided on by the specific application. Generally, quantitative procedures need to focus more on compound separation and may be slightly longer or at least employ more gradual solvent composition gradients.

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Chapter 6

Synthetic Opioid Analysis by LC-MS/MS

David M. Garby and Lynn A. Cheryk

Abstract

Determination of urinary drug concentrations can prove to be very useful in the monitoring of patients who are receiving a variety of synthetic opioids. Through the use of liquid chromatography-tandem mass spectrometry, the simultaneous measurement of synthetic opioids provides for smaller sample volume requirements along with gains in throughput and an increase in the sensitivity of the analysis.

Key words: Opioids, Liquid chromatography-tandem mass spectrometry, Tramadol, Fentanyl, Meperidine, Methadone

1. Introduction

Synthetic opioids are commonly prescribed drugs used for sedation and in the management of pain (1). Many of the synthetic opioids are as effective at inducing analgesia as traditional opiates such as morphine, but have the additional advantages of having fewer toxic side effects and they can often be used at lower therapeutic doses than traditional opiates (1). The synthetic opioids and their respective metabolites, which may also demonstrate analgesic properties, include tramadol and O-desmethyltramadol, fentanyl and norfentanyl, meperidine and normeperidine, as well as methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (1–4). Methadone, with limited use as an analgesic, is primarily used as substitution therapy for opiate dependence and in the treatment of withdrawal symptoms in these patients (4).

Determination of the concentration of opioids in urine is useful in the monitoring of patients under palliative care who often receive various opioids in combination as well as chronic pain management and opiate dependence patients in whom polymedication, compliance, and diversion of medication can be confounding factors (1). The use of a rapid, highly automated, sensitive, and specific analysis

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such as that obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be extremely useful in the monitoring of patient populations such as these. Analysis by LC-MS/MS also offers the added benefit of the simultaneous determination of several drugs at the same time.

2. Materials

2.1. Commercial Standards and Controls

1. Commercial stock standards: Cis-tramadol HCl, O-desmethyl-cis-tramadol HCl, meperidine, fentanyl, norfentanyl, (±) methadone, EDDP, all at 1.0 mg/mL, and normeperidine at 100 µg/mL (Cerilliant Corporation, Round Rock, TX).
2. Commercial internal standards: Cis-tramadol-^{13}C-d_3 HCl, meperidine-d_4, normeperidine-d_4, fentanyl-d_5, norfentanyl-d_5, all at 100 µg/mL, and methadone-d_9 and EDDP-d_3 at 1.0 mg/mL (Cerilliant Corporation, Round Rock, TX).
3. Commercial quality control: Urine Toxicology Control Pain Management 100 (PM 100) (UTAK Laboratories, Valencia, CA).

2.2. Standard Preparation

1. Intermediate fentanyl/norfentanyl stock standard (100 µg/mL): Into a 10 mL volumetric flask, pipette 1.0 mL of fentanyl standard and 1.0 mL of norfentanyl standard. Bring to volume with methanol and mix well.
2. Stock standard 1: Into a 50 mL volumetric flask pipette 0.5 mL each of fentanyl/norfentanyl (intermediate stock standard), tramadol, O-desmethyltramadol, and meperidine; 5.0 mL each of normeperidine, methadone, and EDDP. Bring to volume with methanol and mix well. Final concentrations are 1,000 ng/mL fentanyl and norfentanyl; 10,000 ng/mL tramadol, O-desmethyltramadol, meperidine, and normeperidine; and 100,000 ng/mL methadone and EDDP.
3. Stock standard 2: Into a 10 mL volumetric flask, pipette 1.0 mL of stock standard 1. Bring to volume with methanol and mix well. Final concentrations are 100 ng/mL fentanyl and norfentanyl; 1,000 ng/mL tramadol, O-desmethyltramadol, meperidine, and normeperidine; and 10,000 ng/mL methadone and EDDP.
4. Working standards—*Note*: Working standards 2–6 are calibration standards; Working standard 1 is a zero standard consisting solely of the normal human urine preparation (see Note 1). Prepare each working standard in a separate 200 mL volumetric flask. To each flask, add the amount of stock standard or working standard shown in Table 1. Bring to volume with normal human urine and mix well. Final concentrations are shown in Table 2 (see Note 2).

Table 1
Preparation of working standards

Working standard	Stock standard 1	Stock standard 2	Working standard 5
6	4.0	–	–
5	2.0	–	–
4	1.0	–	–
3	–	2.0	–
2	–	–	2.0
1	–	–	–

Table 2
Analyte concentrations in working standards

	Working standard concentration (ng/mL)					
Analyte	**6**	**5**	**4**	**3**	**2**	**1**
Fentanyl	20	10	5	1	0.1	0
Norfentanyl	20	10	5	1	0.1	0
Tramadol	200	100	50	10	1	0
O-desmethyltramadol	200	100	50	10	1	0
Meperidine	200	100	50	10	1	0
Normeperidine	200	100	50	10	1	0
Methadone	2,000	1,000	500	100	NA	0
EDDP	2,000	1,000	500	100	NA	0

2.3. Internal Standard Preparation

1. Fentanyl-d_5/norfentanyl-d_5 intermediate stock standard (10 µg/mL): Into a 10 mL volumetric flask, pipette 1.0 mL each of fentanyl-d_5 and norfentanyl-d_5 commercial standards. Bring to volume with methanol and mix well.
2. Working internal standard: Into a 100 mL volumetric flask pipette 1.0 mL each of fentanyl-d_5/norfentanyl-d_5 (intermediate stock standard), tramadol-^{13}C-d_3, meperidine-d_4, normeperidine-d_4, methadone-d_9, and EDDP-d_3. Bring to volume with methanol and mix well. Final concentrations are 100 ng/mL fentanyl-d_5 and norfentanyl-d_5; 1,000 ng/mL tramadol-^{13}C-d_3, meperidine-d_4, and normeperidine-d_4; and 10,000 ng/mL methadone-d_9 and EDDP-d_3.

2.4. QC Preparation

1. QC stock standard 1: Into a 50 mL volumetric flask pipette 0.5 mL each of fentanyl/norfentanyl (intermediate stock standard), tramadol, O-desmethyltramadol, and meperidine, and 5.0 mL each of normeperidine, methadone, and EDDP. Bring to volume with methanol and mix well. Final concentrations are 1,000 ng/mL fentanyl and norfentanyl; 10,000 ng/mL tramadol, O-desmethyltramadol, meperidine, and normeperidine; and 100,000 ng/mL methadone and EDDP.
2. Elevated level QC: Into a 200 mL volumetric flask, pipette 3.0 mL of QC stock standard 1 and bring to volume with normal human urine. Final concentrations are 15 ng/mL fentanyl and norfentanyl; 150 ng/mL tramadol, O-desmethyltramadol, meperidine, and normeperidine; and 1,500 ng/mL methadone and EDDP.
3. Low level QC: Into a 200 mL volumetric flask, pipette 300 µL of QC stock standard 1 and bring to volume with normal human urine. Final concentrations are 1.5 ng/mL fentanyl and norfentanyl; 15 ng/mL tramadol, O-desmethyltramadol, meperidine, and normeperidine; and 150 ng/mL methadone and EDDP.

2.5. Supplies and Equipment

1. Analytical column: Kinetex C18, 4.6 × 100 mm, 2.6 µm, Cat# OOD-4462-E0 (Phenomenex, Torrance, CA).
2. SecurityGuard Guard Cartridge Kit, Cat# KJO-42082 (Phenomenex, Torrance, CA).
3. C18 Guard Cartridge, 4.0 × 2.0, Cat# AJO-4286 (Phenomenex, Torrance, CA).
4. Applied Biosystems API 5000™ Tandem Mass Spectrometer with Electrospray Ionization Source (ESI) (AB Sciex, Foster City, CA).
5. Cohesive Technologies Aria™ TLX Series HTLC™ System (high throughput liquid chromatography system) (Thermo Scientific, Franklin, MA).

2.6. Reagents

Note: All reagents listed are HPLC grade or better.

1. Mobile Phase A (0.1% formic acid in deionized water, MP-A): Pipette 2.0 mL of formic acid into 2 L of deionized water. Mix well and store at room temperature.
2. Mobile Phase B (0.1% formic acid in 25/75 acetonitrile/methanol (v/v), MP-B): Combine 0.5 L of acetonitrile with 1.5 L of methanol. Pipette 2.0 mL of formic acid, mix well, and store at room temperature.

3. Methods

The analytical measurement range for methadone and EDDP is tenfold higher than the rest of the analytes of the method. As a result this LC-MS/MS method was divided into two groups of analytes in order to achieve the lower limit of quantification required for most analytes and the upper reporting limit required for methadone and EDDP. Group 1 includes tramadol (see Note 3), O-desmethyltramadol (see Note 4), meperidine, normeperidine, fentanyl, and norfentanyl (see Fig. 1). Group 2 includes methadone and EDDP (see Fig. 2).

3.1. Sample Preparation for Group 1

1. Align the A1 corners of a 96-well protein crash plate and a 96-well 1 mL collection plate. Attach the protein crash plate to the collection well with tape.
2. Pipette 100 µL of each working standard, QC, and patient urine sample into the corresponding well of the protein crash plate.
3. Pipette 25 µL of working internal standard into each well.

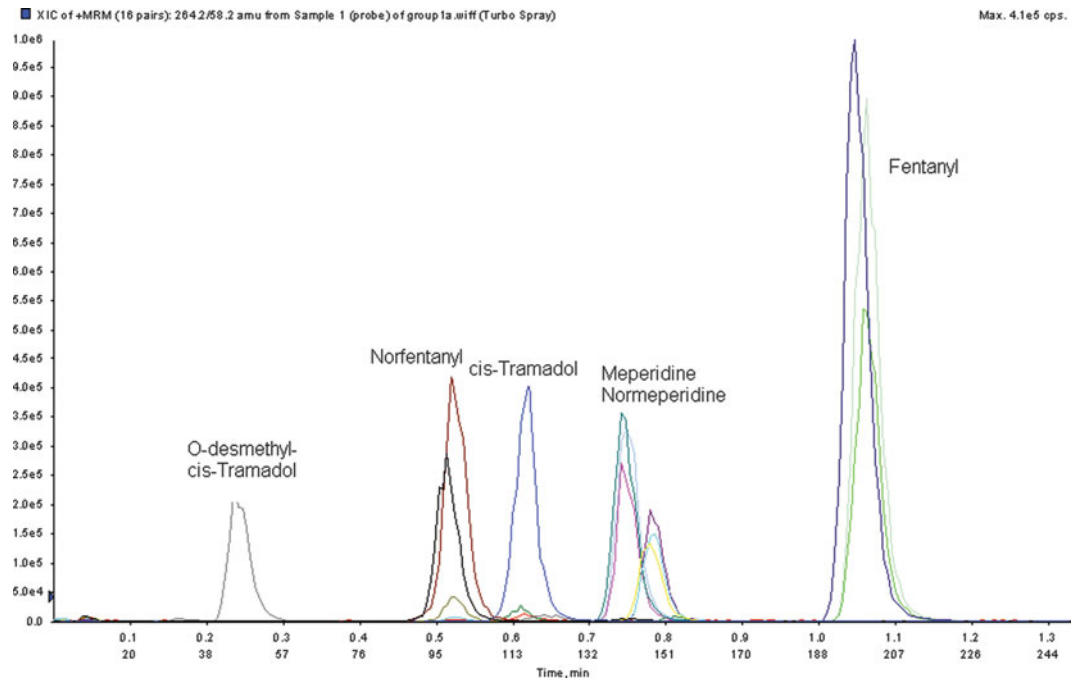

Fig. 1. Chromatogram demonstrating elution of group 1 analytes.

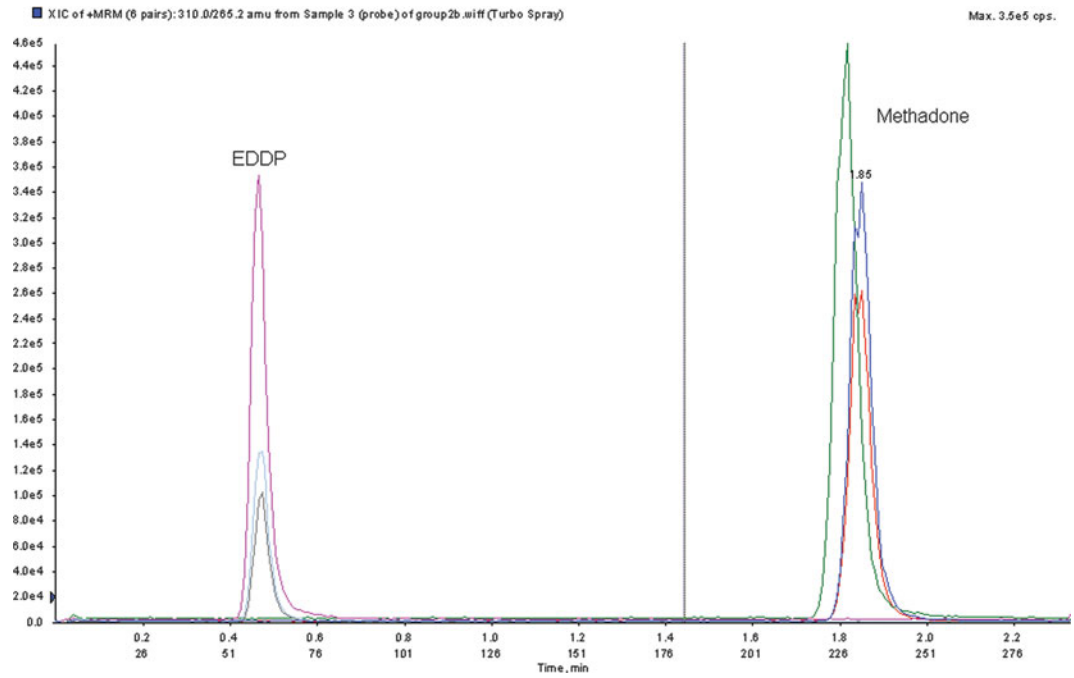

Fig. 2. Chromatogram demonstrating elution of group 2 analytes.

4. Cover the protein crash plate with a 96-well capmat and vortex for approximately 10 s.
5. Place on orbital rotator for 10 min at medium speed (approximately 100 rpm).
6. Remove capmat and pipette 250 µL of acetonitrile to each well.
7. Cover protein crash plate with a capmat and vortex for approximately 10 s.
8. Place on positive pressure manifold and apply nitrogen gas (approximately 5 psi) for approximately 2 min. Collect the supernatant into the 1 mL 96-well collection plate.
9. Transfer 100 µL of the supernatant to the corresponding well of a 2 mL 96-well collection plate.
10. Pipette 500 µL of deionized water into each well.
11. Cover the 2 mL 96-well collection plate with a pierceable template film.

3.2. Sample Preparation for Group 2

1. Align the A1 corners of a 96-well protein crash plate and a 96-well 1 mL collection plate. Attach the protein crash plate to the collection well with tape.
2. Pipette 50 µL of each working standard, QC, and patient urine sample to the corresponding well in the protein crash plate.
3. Pipette 25 µL of working internal standard into each well.

4. Cover the protein crash plate with a 96-well capmat and vortex for approximately 10 s.
5. Place on orbital rotator for 10 min at medium speed (approximately 100 rpm).
6. Remove capmat and pipette 500 µL of acetonitrile to each well.
7. Cover protein crash plate with a capmat and vortex for approximately 10 s.
8. Place on positive pressure manifold and apply nitrogen gas (approximately 5 psi) for approximately 2 min. Collect the supernatant into the 1 mL 96-well collection plate.
9. Transfer 50 µL of the supernatant to the corresponding well in a 2 mL 96-well collection plate.
10. Pipette 2 mL of deionized water into each well.
11. Cover the 2 mL 96-well collection plate with a pierceable template film.

4. LC Method

1. Injection volume: 10 µL.
2. Flow rate: 0.45 mL/min.
3. Mobile phase gradient:
   0–0.25 min: Hold at 7% MP-B.
   0.25–2.25 min: Linear gradient to 55% MP-B.
   7 min: Hold at 55% MP-B.
   7–9 min: Equilibrate at 7% MP-B.

4.1. MS Parameters (Refer to Refs. (1–8))

1. Acquistion windows:
   Group 1
   Data window start: 4 min.
   Data window duration: 1.5 min.
   Group 2
   Data window start: 4.5 min.
   Data window duration: 2.5 min.
2. Collision gas (CAD): 7.0.
3. Curtain gas (CUR): 20.
4. Ion source gas 1 (GS1): 55.
5. Ion source gas 2 (GS2): 55.
6. Ion spray voltage: 5,500.
7. Temperature: 550.
8. Compound-specific parameters are shown in Table 3.

Table 3
Compound-specific parameters

Analyte	Q1 mass (amu)	Q3 mass (amu)	Time (ms)	Declustering potential (DP)	Collision energy (CE)	Cell exit potential (CXP)
Tramadol quantifier	264.2	58.2	5	150	15	14
Tramadol qualifier	264.2	246.4	5	40	15	14
Tramadol-^{13}C-d_3	270.2	58.2	5	40	24	14
O-desmethyltramadol	251.1	58.2	5	20	23	14
Meperidine quantifier	248.0	220.1	5	20	23	14
Meperidine qualifier	248.0	174.2	5	20	23	14
Meperidine-d_4	252.2	224.2	5	50	24	14
Normeperidine quantifier	234.0	160.1	5	50	15	14
Normeperidine qualifier	234.0	56.0	5	50	31	14
Normeperidine-d_4	238.0	58.0	5	50	33	14
Methadone quantifier	310.0	265.2	5	50	24	14
Methadone qualifier	310.0	105.0	5	50	22	14
Methadone-d_9	319.0	268.3	5	50	35	14
EDDP quantifier	278.0	249.3	5	50	28	14
EDDP qualifier	278.0	234.2	5	50	38	14
EDDP-d_3	281.1	249.2	5	50	60	14
Fentanyl quantifier	337.3	188.1	5	60	38	14
Fentanyl qualifier	337.3	105.0	5	60	32	14
Fentanyl-d_5	342.2	188.1	5	50	25	14
Norfentanyl quantifier	233.3	84.1	5	50	25	14
Norfentanyl qualifier	233.3	56.0	5	50	26	14
Norfentanyl-d_5	238.3	84.0	5	50	52	14

5. Notes

1. Remember that carryover should always be investigated during method development by running zero standard blanks after the high standards to insure that there is no carryover present in the system.

2. Working standard 2 is not used to calibrate methadone or EDDP because the relevant analytical range for these two compounds is higher than that of the other opioids. Working standard 3 is the lowest non-blank calibrator for these two analytes.
3. Please note that the tramadol qualifier has a very low intensity and will not give sufficient signal/noise ratio at low concentrations.
4. Due to the absence of an internal standard for the O-desmethyltramadol analyte, it is recommended to report this analyte qualitatively (present/not present).

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Chapter 7

The Determination of Cannabinoids Using Liquid Chromatography with Mass Spectrometric Detection

Oscar Quintela and Dennis J. Crouch

Abstract

Because of their prevalence in drugged driving and other medicolegal investigations, cannabinoids are routinely analyzed by forensic laboratories. Until relatively recently, these analyses were performed by gas chromatography coupled to mass spectrometry (GC-MS). However, the need for derivatization and extensive sample preparation made GC-MS approaches tedious and time consuming. As a consequence, many laboratories have explored alternative analysis techniques. The advent of more affordable liquid chromatography-mass spectrometry (LC-MS) instruments and the utility of atmospheric pressure ionization sources have made LC-MS a promising alternative to GC-MS for the detection and quantitation of cannabinoids in forensic applications.

Key words: Cannabinoids, THC, THC-COOH, LC-MS/MS

1. Introduction

Marijuana (*Cannabis sativa*) is one of the most commonly used and abused substances worldwide. Therefore, reliable analyses for Δ^9-tetrahydrocannabinol (THC) and its characteristics metabolites are needed for testing indications such as the workplace, drug-treatment monitoring, drug courts, probation, and parole and for testing drivers suspected of operating motor vehicles while under the influence of drugs (DUID).

During the last decade, the use of liquid chromatography-mass spectrometry (LC-MS) technology has been increasing in clinical and forensic laboratories. This expansion is in part because of the gradual introduction of relatively low-cost LC-MS instruments and the availability of robust atmospheric pressure ionization (API) sources. The combination of liquid chromatographic separation and mass spectrometric detection provides the laboratory an efficient

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and reliable alternative to gas chromatography-mass spectrometry (GC-MS) for the determination of THC and its metabolite 11-nor-Δ^9-carboxy-tetrahydrocannabinol (THC-COOH), and is the focus of the following chapter.

This chapter is divided into the three major processes required for the analysis of THC and its major metabolites: *sample preparation*, *separation*, and *detection*. The authors present their own methods in detail and outline contributions from other researchers (summarized in Table 1) to broaden the scope of the chapter.

1.1. Sample Preparation

Various extraction procedures have been used to isolate THC and its metabolites from biological matrices. The selection of liquid–liquid (LLE) or solid-phase (SPE) extraction depends on a laboratory's experience and instrumentation: LLE is less expensive and achieves reasonable recoveries of most cannabinoids; however, the expanding array of SPE sorbents should be considered especially if the chemical "cleanness" of the extraction residue is imperative (e.g., analysis of THC in hair). Also, SPE can be readily automated or placed online with the LC-MS/MS, which can reduce analyst variability and solvent handling while increasing laboratory throughput (22). Although extraction is usually performed prior to instrumental analysis, recently Chebbah et al. (20) presented a method for use in doping control applications in which THC-COOH was quantified in the urine without sample extraction.

LLE has been the traditional method for isolating many drugs from biological matrices, based on partitioning of the drugs between the aqueous phase (biological) and an organic extraction solvent. The pH of the aqueous phase can be adjusted such that the drugs are unionized and readily extracted into the organic solvent. Several organic solvents can be used to extract cannabinoids, including hexane, methanol–chloroform (9:1, v/v) (23), diethylether:ethylacetate (1:1, v/v) (8), acetonitrile:ethylacetate (1:1, v/v) (3); and hexane:ethyl acetate (9:1, v/v) (24). This last method has been modified by the authors of this chapter (9) to isolate THC and THC-COOH from a variety of biological matrices such as blood, plasma, urine, and oral fluid.

SPE has several advantages over LLE: no emulsions, less solvent use, improved recoveries, and chemically cleaner extracts. The choice of sorbents and selectivity of the organic solvent mixtures used as eluents give SPE considerably more versatility than LLE for the extraction of THC and its metabolites.

Nonpolar silica sorbents such as C18 have been used extensively (4). Elian and Hackett (19) reported using a novel fluorinated SPE sorbent for isolation of THC and THC-COOH from whole blood. In a totally automated SPE method Hysphere C8-EC extraction columns were used (16–18, 21), which allowed the direct introduction of extracted cannabinoids into the LC column.

Mixed-mode bonded silica sorbents have silanol groups that are partially derivatized with octyl chains of medium length,

Table 1
Characteristics for LC-MS(MS) analysis from selected references

			Chromatography conditions					MS		
Analytes	Matrix	Extraction	Column	Dimensions (mm); particle size (μm)	Mode	Mobile Phase	Run time (min)	Apparatus	Ionization	Reference
THC-COOH	Urine	SPE (C18)	Xterra-MS C18	3.9×20; 3.5	Gradient	A: 1 mM ammonium formate/0.1% formic acid, pH 3.2 B: Methanol	6.5	QqQ	APCI–	(1)
THC-COOH-glucuronide	Plasma, urine	LLE and SPE (Certify)	Zorbax Eclipse XDB C8	2.1×150; 5	Isocratic	20 mM ammonium acetate pH 4.0/ methanol/ acetonitrile (41:41:18, by volume)	7	QqQ	ESI+	(2)
THC, THC-COOH, 11-OH-THC	Plasma	LLE	Symmetry C18	2.1×150; 5	Isocratic	A: 2 mM ammonium acetate B: Acetonitrile	30	Q	ESI–	(3)
THC, THC-COOH, 11-OH-THC; THC-COOH-glucuronide	Plasma	SPE (C18)	Luna PhenylHexyl	2×50; 3	Gradient	A: 5 mM ammonium acetate, pH 6.5 B: Acetonitrile	11	QqQ	ESI+	(4)
THC	Oral fluid	LLE	XTerra MS C18	2.1×100; 3.5	Isocratic	0.1% Formic acid/ acetonitrile (15:85, v/v)	5	Q	ESI+	(5)

(continued)

Table 1
(continued)

			Chromatography conditions					MS		
Analytes	**Matrix**	**Extraction**	**Column**	**Dimensions (mm); particle size (μm)**	**Mode**	**Mobile Phase**	**Run time (min)**	**Apparatus**	**Ionization**	**Reference**
8-THC, Δ8-THC-COOH	Plasma	LLE	Symmetry C18	2.1×150; 5	Gradient	A: 2 mM ammonium acetate with 5% acetonitrile B: 5% of 2 mM ammonium acetate in acetonitrile	30	Q	ESI−	(6)
THC	Oral fluid	LLE	XTerra MS C18	2.1×150; 3.5	Isocratic	1 mM ammonium formate/methanol (10:90, v/v)	8	QqQ	ESI+	(7)
THC, THC-COOH, 11-OH-THC, CBN, CBD	Plasma	LLE and SPE (Certify II)	Synergi MAX-RP C12	2×75; 4	Gradient	A: 10 mM ammonium formate, pH 3.0 B: 10% 10 mM ammonium formate, pH 3.0 in acetonitrile	25	IT	APCI+	(8)
THC, THC-COOH	Oral fluid	LLE	XTerra MS C18	2.1×150; 3.5	Gradient	A: 10 mM ammonium formate, pH 3.5 B: Methanol	19	QTOF	ESI+	(9)

THC, THC-COOH	Oral fluid, urine, blood	SPE (Certify; CleanScreen CSTHC)	Symmetry C18	2.1×150; 5	Isocratic	0.1% Formic acid/acetonitrile (30:70, v/v)	15	Q	ESI+ and –	(10)
THC, THC-COOH, 11-OH-THC	Blood	SPE (Certify)	BEH C18	2.1×100; 1.7	Gradient	A: 0.1% Formic acid, pH 2.6 B: Acetonitrile	10	QqQ	ESI+	(11)
THC-COOH	Urine	No extraction	BEH C18	1×100; 1.7	Gradient	A: 0.1% Formic acid, pH 2.8 B: Acetonitrile	4.2	QqQ	ESI–	(12)
THC, THC-COOH, 11-OH-THC	Blood	LLE	XBridge C18	2.1×150; 3.5	Isocratic	0.1% Formic acid/methanol (20:80, v/v)	13	QqQ	ESI+	(13)
THC, THC-COOH, 11-OH-THC	Blood	SPE (Polychrom THC)	Zorbax Extend C18	2.1×50; 1.8	Gradient	A: 20 mM ammonium formate, pH 8.6 B: Methanol	14	QqQ	ESI+	(14)
THC	Hair	SPE (Polychrom THC)	Zorbax Extend C18	2.1×50; 1.8	Gradient	A: 20 mM ammonium formate, pH 8.6 B: Methanol	6	QqQ	ESI+	(15)
THC, THC-COOH, 11-OH-THC, CBN, CBD	Blood	Online SPE (Hysphere C8)	XTerra MS C18	3×20; 5	Gradient	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile	10	QqQ	ESI+	(16)
THC-COOH	Urine	Online SPE (Hysphere C8)	Atlantis dC18	3×100; 3	Gradient	A: 0.1% Formic acid in water B: Acetonitrile	6	QqQ	ESI+	(17)

(continued)

Table 1
(continued)

			Chromatography conditions					MS		
Analytes	**Matrix**	**Extraction**	**Column**	**Dimensions (mm); particle size (μm)**	**Mode**	**Mobile Phase**	**Run time (min)**	**Apparatus**	**Ionization**	**Reference**
THC-COOH	Urine	Online SPE (Hysphere C8)	XBridge C8	2.1 × 50; 3.5	Gradient	A: 0.2% Formic acid in water B: Acetonitrile	6	QqQ	ESI+	(18)
THC, THC-COOH	Blood	SPE (Fluorinated C10)	Imtakt US-C18	2 × 50; 5	Gradient	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile	5.5	QqQ	ESI+ (THC); ESI− (THC-COOH)	(19)
THC-COOH	Urine	No extraction	Zorbax RX-C8	2.1 × 150; 5	Gradient	A: 1 mM ammonium acetate/ 0.001% acetic acid B: Methanol with 1 mM ammonium acetate/0.001% acetic acid	11.5	QqQ	ESI + and −	(20)
THC, THC-COOH, 11-OH-THC, CBN, CBD	Urine	Online SPE (Hysphere C8)	XTerra MS C18	3 × 20; 5	Gradient	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile	10	QqQ	ESI+	(21)

Abbreviations: *11-OH-THC* 11-hydroxy-Δ^9-tetrahydrocannabinol, *APCI* atmospheric pressure chemical ionization, *CBD* cannabidiol, *CBN* cannabinol, *ESI* electrospray ionization, *IT* ion trap mass analyzer, *Q* single quadrupole analyzer, *QqQ* triple quadrupole mass spectrometer, *QqTOF* quadrupole time-of-flight mass spectrometer, *THC* tetrahydrocannabinol, *THC-COOH* 11-nor-Δ^9-carboxy-tetrahydrocannabinol

but retain their cation or anion exchange functionalities. In particular, strong anion exchange (SAX) sorbents have been optimized for the extraction of acidic drugs such as THC-COOH, which possesses both nonpolar and anionic character. Several authors (10, 11) have also reported using strong cation exchange columns (SCX) for the extraction of cannabinoids from blood, urine, and oral fluid.

SPE copolymers are the result of the polymerization of two monomers, such as styrene and divinylbenzene. These sorbents have several potential advantages when compared to the classic silica-based sorbents, including increased sorbent capacity for smaller cartridge sizes, use under virtually any pH conditions, and the ability to remain "wetted," thereby avoiding the reduced recovery associated with drying of traditional sorbents. Like silica-based sorbents, polymeric sorbents can be modified by adding cation or anion exchange groups on their surface. Thus, highly specific extraction methods for cannabinoids have been developed for use with biological fluids (14) or hair (15).

1.2. Separation

Recently several studies have reported the use of analytical columns packed with sub-2 μm size particles (11, 12, 14, 15). These columns are especially designed for use with ultrahigh-performance liquid chromatography (UHPLC). The smaller particles are more efficient because they can be used at higher linear velocities, which provides both better resolution and shorter analysis times. UHPLC methods have been reported using isocratic and gradient elutions, a variety of mobile phases, and aqueous buffers with pH ranging from 2.6 to 8.6 to ensure optimum ionization and intensities for specific mass spectrometer ionization sources (14).

Because of the lipophilicity of most cannabinoids and their affinity for reverse-phase columns, a high percentage of organic solvent in the mobile phase is needed to elute THC and its metabolites. It is important to note that most reported retention times for cannabinoids were generally the result of a balance between separation of the analytes, separation from possible matrix interferences, and acceptable analysis times. Too short of an analysis time may lead to chromatographic challenges such as interferences or ion suppression (19). However, rapid methods may be acceptable if the mass spectrometer is capable of efficient switching between negative to positive modes, to minimize the need for chromatographic separation of THC from various metabolites.

1.3. Detection

The selectivity of tandem mass spectrometers reduces the need for extensive sample preparation (25). However, insufficient sample preparation for LC-MS/MS analyses may result in matrix effects such as ion suppression. Therefore, it is essential that appropriate steps be taken during method validation to ensure that the sample preparation procedure removes potential interferences and minimizes matrix effects (26).

Compared to many drugs of toxicological interest such as basic drugs, cannabinoids are not as efficiently ionized by either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). As shown in Table 1, ESI has been the most widely reported ionization technique for the analysis of THC. THC-COOH, though poorly ionized, may be ionized and detected by either positive or negative techniques with similar efficiency and sensitivity. Selection of the most suitable ionization technique is predicated upon the target cannabinoid's structure, experimental conditions (type of matrix, presence of interferences, etc.), and performance of the source—which may differ by manufacturer. Several source parameters must be optimized for the efficient ionization of the cannabinoids, most notably source temperature and gas flows, as well as capillary voltage (in ESI) or corona discharge (in APCI).

The three processes discussed in this chapter are interactive and iterative. Initially, one should (1) optimize the mass spectrometry ionization and detection parameters and LC separation conditions and (2) apply the method to biological samples to ensure that there are no interferences from the extraction or the matrix. Re-optimization may then be necessary to ensure the most accurate and reliable analyses.

2. Materials

Various SPE column configurations and sorbents can be used (Table 1). Extraction manifolds or automatic extraction apparatuses such as robotics can be used. Depending on the instrumentation available, an integrated online SPE unit can be used for analyte isolation and sample extraction.

2.1. Standard Reagents

1. THC, THC-COOH, D_3-THC, and D_3-THC-COOH are available commercially as 1.0 mg/mL methanolic stocks (Cerilliant, Round Rock, TX) (see Note 1).
2. Working standard solutions:
   (a) 500 ng/mL spiking standard: To a 10 mL volumetric flask, add 100 μL of each 1.0 mg/mL stock standard (THC and THC-COOH), bring to volume with methanol, and mix well. Quantitatively transfer 250 μL to a 5 mL volumetric flask, bring to volume with methanol, and mix well. Store at −20°C and in the dark, but allow to warm to room temperature prior to use.
   (b) 100 ng/mL spiking standard: Quantitatively transfer 1 mL of the 500 ng/mL spiking standard to a 5 mL volumetric flask, bring to volume with methanol, and mix well.

Table 2
Preparation of calibration standards

Calibrator number	THC and THC-COOH concentration (ng/mL)	Volume blank matrix (μL)	Working standard solution (ng/mL)	Volume working standard solution (μL)
1	0.1	500	1	50
2	0.2	500		100
3	0.5	500	5	50
4	1	500		100
5	2.5	500	25	50
6	5	500		100
7	10	500	100	50
8	20	500		100
9	50	500	500	50
10	100	500		100

(c) 25 ng/mL spiking standard: Quantitatively transfer 250 μL of the 500 ng/mL spiking standard to a 5 mL volumetric flask, bring to volume with methanol, and mix well.

(d) 5 ng/mL spiking standard: Quantitatively transfer 1 mL of the 25 ng/mL spiking standard to a 5 mL volumetric flask, bring to volume with methanol, and mix well.

(e) 1 ng/mL spiking standard: Quantitatively transfer 200 μL of the 25 ng/mL spiking standard to a 5 mL volumetric flask, bring to volume with methanol, and mix well.

3. Calibration standards: Each calibrator is made by spiking 500 μL of the appropriate blank matrix according to Table 2. Calibrators should be made fresh for each run.
4. 100 ng/mL working internal standard: To a 10 mL volumetric flask, add 100 μL of each 1.0 mg/mL stock standard (D_3-THC and D_3-THC-COOH), bring to volume with methanol, and mix well. Quantitatively transfer 100 μL to a 10 mL volumetric flask, bring to volume with methanol, and mix well. Store at −20°C and in the dark, but allow to warm to room temperature prior to use.

2.2. Sample Preparation

High-purity hexane, ethyl acetate, and methanol should be used, and water that has a resistivity of ≤18.2 MΩ-cm. It is highly recommended that all glassware be silanized (see Note 2). Mixtures of aqueous solvents with organic solvents should be prepared freshly before their use (methanol, deionized water, and ammonium hydroxide).

1. Mobile Phase A (MP-A): 10 mM aqueous ammonium formate, pH 3.5. To a 1 L beaker, add approximately 750 mL of water. While stirring, slowly add 0.63 g of ammonium formate and mix to dissolution. Bring to volume with water and add dilute formic acid to adjust pH to 3.5.
2. Mobile Phase B (MP-A): 100% Methanol.
3. 0.2 N sodium hydroxide: To a 1 L volumetric flask, add about 750 mL of Type I water. While stirring, slowly add 8 g of sodium hydroxide. Allow to cool before bringing to volume with Type I water. Store at room temperature for 1 month.
4. Hexane:ethyl acetate (9:1, v/v): In a beaker, combine 90 mL of hexane and 10 mL of ethyl acetate, and mix. Prepare fresh weekly and store at 4°C.
5. 6 M hydrochloric acid: To a 250 mL beaker add 125 mL of concentrated HCl (37.2%, 12.1 M) using a 250 mL graduated cylinder. While stirring continuously, very slowly add 125 mL of Type I water using a 250 mL graduated cylinder. The mixing beaker may be immersed in a bucket of ice to control the solution temperature. Store at room temperature for 6 months.
6. 0.1 M hydrochloric acid: To a 500 mL volumetric flask, add 8.3 mL of 6 M HCl using a glass pipette. Bring to volume with Type I water. Store at room temperature for 1 month.

2.3. Sample Analysis

1. For LC separation, various reverse-phase analytical columns can be used (Table 1). Most commonly, C18 columns are used. The LC column used for the analytical method described below is an XTerra C_{18} MS, 3.5 µm (150×2.1 mm I.D.) reversed-phase column (Waters, Milford, MA).
2. An Applied Biosystems QStar® XL (QTOF, quadrupole—time of flight) instrument or a triple quadrupole (QqQ) can be used for the method described here.

3. Methods

3.1. Sample Preparation and Sample Extraction

The purpose of sample preparation procedure(s) is to isolate the compounds of interest (THC and its metabolites) from the biological matrix and, if necessary, to concentrate the analytes to facilitate detection. Either LLE or SPE can be used for extracting cannabinoids from biological matrices. Below are detailed the chosen LLE.

1. Add 500 µL of blank matrix to labeled and silanized glass tubes for calibration standards. Blood, plasma, or urine are added neat; oral fluid is first diluted 1:1 with Mili-Q water. Add 500 µL of unknown samples into similar tubes.

2. Add 1 mL of 0.2 N sodium hydroxide to each tube containing blank matrix or unknown samples.
3. Add the appropriate volumes of the THC and THC-COOH working solutions as shown in Table 2 to the tubes containing blank matrix.
4. Add 75 μL of the internal standard working solution to each tube and vortex-mix for 30 s.
5. Add 3 mL of 9:1 hexane:ethyl acetate to each tube and mix the tubes for 30 min at approximately 60 rpm on a reciprocating shaker.
6. Centrifuge for 5 min at approximately 2,000 × *g*.
7. Transfer the organic layer, containing the neutral and basic compounds (i.e., THC), to a fresh tube for THC analysis (see step 8). Save the basic (aqueous phase) containing acidic compounds used for the measurement of THC-COOH (see step 9).
8. Fraction containing THC:
   (a) Add 2 mL of 0.1 N HCl to each tube.
   (b) Shake the tubes for 15 min and then centrifuge for 15 min as above.
   (c) Transfer the organic layer into a fresh silanized conical tube and prepare for evaporation (see below).
9. Fraction containing THC-COOH:
   (a) Add 500 μL of 6 M HCl to the basic 0.2 N NaOH layer from the initial extraction (step 7) and 3 mL of 9:1 hexane:ethyl acetate to each tube.
   (b) Shake the tubes for 30 min and centrifuge for 15 min as above.
   (c) Combine the organic layer from this extraction with the organic layer from the THC extraction.
10. Evaporate the combined solvents to dryness at 40°C.
11. Reconstitute the dried extracts in 50 μL of Mobile Phase B (see Note 3).

3.2. LC-MS/MS Analysis

1. Chromatography conditions

   C8 or C18 reverse-phase LC chromatography columns and guard columns with similar packing material have been predominantly used for the analysis of cannabinoids extracted from biological matrices.
2. Injection volume: 20 μL.
3. Autosampler temperature: Preferably at refrigerated.
4. Column temperature: 30°C.
5. LC flow rate: 200 μL/min.

6. Mobile phase gradient:
   0.0–0.2 min: Hold at 23% of MP-B.
   0.2–6.0 min: Linear gradient to 96% of MP-B.
   6.0–9.0 min: Hold at 96% of MP-B.
   9.0–10.5 min: Linear gradient to 23% MP-B.
   10.5–19.0 min: Re-equilibrate at 23% MP-B.

3.3. Source Parameters

1. Ionization mode: Positive.
2. ESI capillary voltage: 5,200 V.
3. Auxiliary gas temp.: 400°C.
4. Nebulizer gas (N_2): 45 arbitrary units (arb units).
5. Auxiliary gas (heated gas, N_2): 90 arb units.

3.4. Mass Spectrometric Analysis and Detection (See Note 4)

1. Quadrupole/time-of-flight mass spectrometer (see Note 5): Compound-dependent parameters are detailed in Table 3.
2. When using triple quadrupole mass spectrometer, reported transitions of the cannabinoids in positive ion MRM mode include (see Note 6):
   m/z 315 → 193 and m/z 315 → 259 for THC.
   m/z 345 → 327 and m/z 345 → 299 for THC-COOH.
   Note: If THC-COOH is analyzed in negative mode, the transitions to be monitored are m/z 343 → 299 and m/z 343 → 245.

4. Notes

1. Because of the challenges of extracting and analyzing THC and its metabolites, most methods incorporate deuterium-labeled internal standards. These compounds are chemically quite similar to THC and its metabolites and, therefore, extract, ionize, and can be detected with essential equivalent efficiency as the target analytes. Deuterated analogues such as THC-D_3 and THC-COOH-D_3 are commercially available and are especially recommended for quantification of the cannabinoids.
2. When determining THC and metabolites, adsorptive losses can occur during storage and extraction. Therefore, all glassware should be thoroughly cleaned and silanized.
3. To improve dissolution of THC and its metabolites during reconstitution 100% solvent (methanol or acetonitrile) should be used. If this is not possible, first add the organic solvent, mix thoroughly, and then add the aqueous phase.

Table 3
Optimized MS/MS parameters for the detection of THC, THC-COOH, and their respective deuterated analogues by means of a QTOF instrument

Molecule	Ion selected in Q1 (*m/z*)	Declustering potential (V)	Focusing potential (V)	Declustering potential 2 (V)	Collision energy (V)	Collision gas (arb units)	Scan range in the TOF analyzer (*m/z*)
THC	315.2	40	160	5	27	5	70–335
THC-d_3	318.2	40	160	5	27	5	70–335
THC-COOH	345.2	40	140	10	23	5	100–350
THC-COOH-d_3	348.2	40	140	10	23	5	100–350

4. The mass spectrometric conditions should be optimized for each target analyte. Optimization of ion transmission, collision energy, and collision pressure can be achieved by infusing the target analyte(s) in solution via a syringe pump (typical infusion rates of approximately 10 µL/min flow of a 0.1–1.0 µg/mL analyte solution). Through a systematic review of the collision-induced dissociation product ions of the quasi-molecular ion (Q1) one can select those transitions with the optimum signal-to-noise ratio. To ensure confidence in the identification of THC and its metabolites, at least two transitions should be monitored. The most abundant transition is commonly used for quantification and an alternate transition(s) is used qualitatively to add confidence to the identification. Calculating the ratio of the transitions and comparing it to a calibrator or a selected control are often used to further ensure accurate identification of THC or its metabolites.
5. The authors of this chapter reported an alternate MS technique for determination of THC and THC-COOH (9). A tandem-in-time mass spectrometer (QqTOF) instrument was used. Fragmentation of the precursor ions through low-energy collision-induced dissociation was performed in collision cell in a fashion analogous to a QqQ; however, the product ions were acquired by a TOF mass analyzer. Quantitation of THC and THC-COOH was performed by measuring the ion abundance of one (or two) diagnostic ion in the product ion spectrum. The advantages of QqTOF compared to QqQ are the improved mass resolution and accuracy and the capability to acquire full mass spectra without the loss of sensitivity. In this method, accurate mass measurement was achieved to ±5 ppm. The enhanced mass accuracy of TOF allowed for greater specificity than can be obtained by other tandem MS techniques (such as using selected reaction monitoring mode).
6. QqQ analysis in combination with LC separation has become a technique of choice to detect and quantify trace amounts of THC and its metabolites in biological matrices. Although QqQ instruments can be operated in a number of modes, multiple reaction monitoring (MRM) is often used for the analysis of THC and its metabolites because it satisfies the needs for selectivity and sensitivity.

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Chapter 8

Cocaine and Metabolites by LC-MS/MS

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Abstract

Abuse of the stimulant cocaine (COC) is a common problem in the United States and elsewhere. The drug can be used either as the powder or as the free base (crack COC), and causes feelings of alertness and euphoria; both forms of COC are powerfully addictive. The assay described here is designed to detect and quantitate parent COC, its major metabolite benzoylecgonine, and a selection of metabolites that can provide specific information about sample validity (*m*-hydroxybenzoylecgonine), potential toxicity (norcocaine), route of administration (anhydroecgonine methyl ester), and co-utilization with ethanol (cocaethylene).

Key words: Cocaine, Crack, Benzoylecgonine, Norcocaine, Cocaethylene, *m*-Hydroxybenzoylecgonine, Anhydroecgonine methyl ester, LC-MS/MS

1. Introduction

Cocaine (COC) is an alkaloid found in *Erythroxylon coca* (1, 2), and has been used for over 2,000 years for its stimulant properties. COC blocks synaptic reuptake of the neurotransmitters norepinephrine, dopamine, and serotonin, resulting in a state of alertness and euphoria. Due to the potency of these effects, COC is a powerfully addictive drug with a long history of licit and illicit use in the United States and elsewhere (3). COC can be used in powder form or as the free base (crack COC), and remains one of the most common illicit drugs of abuse (2, 4). According to the 2008 National Survey on Drug Use and Health, overall COC (powder and crack combined) use among individuals aged 12 and older has remained relatively stable since 2002 with 1.9 million users in 2008; however, the use of crack declined notably from previous years (5).

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COC is an ester of benzoic acid and the amino alcohol methylecgonine, with a hydrophobic region including the benzene ring and a hydrophilic region consisting of an amine moiety (1). In the body, COC is rapidly transformed to its major metabolites benzoylecgonine (BE) and ecgonine methyl ester (2) (Fig. 1). Formation of BE can occur via spontaneous hydrolysis at physiological and alkaline pH (2), or by the activity of liver carboxylesterases (6). BE is pharmacologically inactive; however, because the half-life of BE is significantly longer than that of COC, most urine assays for assessing COC use are designed to detect BE.

BE can be converted to minor metabolites such as *m*-hydroxybenzoylecgonine (*m*-HOBE) and *p*-hydroxybenzoylecgonine (1, 7). Of these, *m*-HOBE is useful for determining COC exposure in meconium samples from at-risk newborns, since it has a longer half-life than BE (8, 9). It is also useful in urine analysis: *m*-HOBE is formed exclusively by in vivo metabolism, thus its presence can refute claims that positive BE results are due to adulteration of a urine sample with COC followed by spontaneous hydrolysis to BE (10). In addition, because of its longer half-life relative to BE, *m*-HOBE has the potential to lengthen the detection window for assessment of COC use. N-demethylation of COC produces norcocaine (NC) which can be metabolized further to hepatotoxic compounds (2). NC concentrations are higher in patients with cholinesterase deficiency (11), and in users who simultaneously ingest COC and ethanol (12).

Simultaneous use of COC and other drugs is common, and is most frequently seen with COC and alcohol. With concomitant alcohol use, liver methylesterase catalyzes transesterification of COC to cocaethylene (CE), an active (2), though less potent, metabolite (13). This conversion occurs approximately 3.5 times faster than hydrolysis to BE (14) and prolongs the physiological response; thus, adding ethanol to COC use increases sensations of euphoria and well-being (2, 15). As a result, users may ingest large amounts of COC and ethanol and thus be at greater risk for toxicity than if either drug were used alone. Furthermore, formation of NC is also enhanced by simultaneous administration of COC and ethanol (1, 7, 12). For these reasons, use of both COC and ethanol is associated with 18- to 25-fold higher threat of immediate death relative to the use of COC alone (2).

Use of crack can be specifically determined by the presence of metabolites unique to the thermal degradation that occurs when COC is smoked. Anhydroecgonine methyl ester (AEME, methyl ecgonidine) is found in individuals following crack administration; a related compound, anhydroecgonine ethyl ester (AEEE,

ethyl ecgonidine) has been identified in COC smokers who simultaneously use ethanol (16).

Most methods for detection and/or quantitation of COC and its associated analytes only determine COC and BE, although tests including wider arrays of analytes have been described (17, 18). As an improvement to the existing methods, we describe here a sensitive LC-MS/MS assay that focuses on clinically and forensically significant analytes. There are many additional metabolites of COC, including ecgonine methyl ester and ecgonine ethyl ester, which were not included in this assay as they are not known to influence the evaluation of potential toxicity or route of administration. The following method was developed to detect and quantitate COC, its major metabolite BE, and a selection of metabolites that can provide specific information about sample validity (*m*-HOBE), potential toxicity (NC), route of administration (AEME), and co-utilization with ethanol (CE).

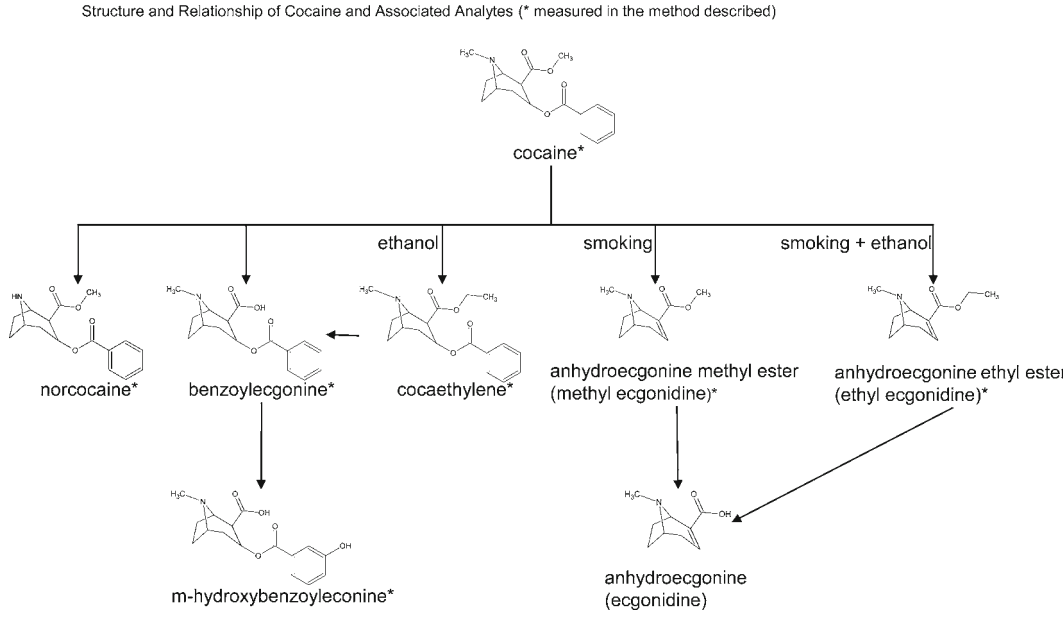


Fig. 1. Chromatography of cocaine and related analytes. A 5 ng/mL standard containing all analytes and internal standards is shown. Anhydroecgonine ethyl ester (AEEE) is shown but not described in the above method because the pure compound is not commercially available. *AEME* anhydroecgonine methyl ester, *BE* benzoylecgonine, *CE* cocaethylene, *COC* cocaine, *m*-HOBE *m*-hydroxybenzoylecgonine, *NC* norcocaine.

2. Materials

2.1. Solvents and Chemicals

1. All reagents are of HPLC or analytical grade.
2. COC, *m*-HOBE, norcocaine, cocaethylene, benzoylecgonine, AEME, and deuterated compounds used as internal standards (BE-d_3, COC-d_3, and CE-d_8) as 1.0 mg/mL (unlabeled compounds) or 100 μg/mL (deuterated compounds) methanolic stocks (Cerilliant, Round Rock, TX).

2.2. Prepared Reagents

1. Extraction solvent: Mix dichloromethane, isopropyl alcohol, and ammonium hydroxide in a volume ratio of 78:20:2. Prepare fresh daily.
2. Mobile Phase A: 20 mM ammonium formate pH 2.7, prepare with Type I water. Add 1.26 g of ammonium formate to 1 L of Type I water. Adjust pH to 2.7 using concentrated formic acid.
3. Mobile Phase B: 50:50 Methanol/acetonitrile.
4. 0.1 M acetic acid buffer. To a 1 L volumetric flask, add about 750 mL of Type I water. While stirring, slowly add 11.6 mL of glacial acetic acid. Allow to cool before bringing to volume with Type I water. Mix and transfer contents to a 2 L reagent bottle. Add another 1 L of Type I water for a final volume of 2 L. Mix well.
5. 1.0 μg/mL spiking standard and spiking control.
   (a) Spiking standard: Quantitatively transfer 25 μL of each 1.0 mg/mL stock standard (AEME, COC, *m*-HOBE, NC, CE, and BE) to a 25.0 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.
   (b) Spiking control: Repeat step 5a using stock standards that have a different lot number, or were prepared independently from the spiking standard stocks.
6. 0.1 μg/mL spiking standard and spiking control.
   (a) Spiking standard: Quantitatively transfer 1.0 mL of the 1.0 μg/mL spiking standard to a 10.0 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.
   (b) Spiking control: Repeat step 6a using the 1.0 μg/mL spiking control.

7. 0.01 μg/mL spiking standard and spiking control.
   (a) Spiking standard: Quantitatively transfer 100 μL of the 1.0 μg/mL spiking standard to a 10.0 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.
   (b) Spiking control: Repeat step 7a using the 1.0 μg/mL spiking control.
8. 0.1 μg/mL Working Internal Standard (IS). Quantitatively transfer 1.0 mL of the 100.0 μg/mL IS stock standards (BE-d_3, COC-d_3, and CE-d_8) to a 100.0 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.

2.3. Supplies and Analytical Equipment

1. Clean Screen® Extraction Columns (United Chemical Technologies, Inc.).
2. Triple quadrupole mass spectrometer using electrospray ionization source, e.g., Agilent Model 6410 or similar (Agilent Technologies).
3. Analytical column: Agilent Rapid Resolution HT XDB-C_8 (50 × 2.1 mm, 1.8 μm).

3. Methods

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

1. To prepare the unextracted control: Add 50 μL of the 0.1 μg/mL spiking standard to a labeled 16 × 100 test tube. Add 50 μL of Working Internal Standard. Add 3 mL extraction solvent. Evaporate to dryness under a gentle nitrogen flow with heat ≤40°C. Reconstitute in 200 μL of Mobile Phase A. Set aside until the remaining samples are ready for analysis (Subheading 3.3).
2. To prepare working calibration standards for the run: Aliquot the appropriate amount of 1.0, 0.1, or 0.01 μg/mL spiking standard into a labeled 16 × 125 mm test tube (see Table 1). Dilute to 1.0 mL with drug-free urine. Aliquot 1.0 mL of drug-free urine for the negative (carryover) control.
3. To prepare working quality controls for the run: Dilute spiking controls to desired concentrations, using procedure similar to preparation of calibration standards (see Table 2). Bring to 1.0 mL volume with drug-free urine.

Table 1
Working standard preparation

Working standards	Volume added from spiking standards		
Final concentration (ng/mL)	**1.0 μg/mL S.S.**	**0.1 μg/mL S.S.**	**0.01 μg/mL S.S.**
1.0			100 μL
2.5			250 μL
5.0		50 μL	
15.0		150 μL	
30.0	30 μL		
50.0	50 μL		
100.0	100 μL		

Table 2
Working control preparation

Working controls	Volume added from spiking controls		
Final concentration (ng/mL)	**1.0 μg/mL S.C.**	**0.1 μg/mL S.C.**	**0.01 μg/mL S.C.**
1.5			150 μL
10.0		100 μL	
75.0	75 μL		

4. To prepare unknown samples: Add 1.0 mL of each sample to appropriately labeled 16×125 mm test tubes (see Note 1 for samples expected to have high values).
5. Add 50 μL of Working IS to all tubes (i.e., standards, controls, and unknowns).
6. Add 3 mL of 0.1 M acetic acid buffer to all tubes.
7. Vortex to mix.

3.2. Extraction

Note—Preconditioning and extraction steps may vary between SPE column manufacturers. Follow recommendations for the column used.

1. Column extraction—Do not allow columns to dry out until stated. Place one labeled column per sample (standard, control, or unknown) in vacuum manifold. Set to low vacuum (1–2 mL/min). Precondition the columns with 3 mL of methanol, followed by 3 mL of 0.1 M acetic acid buffer. Apply each sample

to the appropriate column. Wash the columns with 3 mL of 0.1 M acetic acid buffer, followed by 3 mL of methanol. Increase vacuum to full power and dry columns under full vacuum for 5 min.

2. Return to low vacuum (1–2 mL/min) and place a labeled 16 × 100 glass tube beneath each column.
3. Add 3 mL extraction solvent to each sample and elute into glass tubes.
4. Evaporate to dryness under a gentle nitrogen flow with heat ≤40°C.
5. Reconstitute each sample in 200 µL of Mobile Phase A.
6. Transfer to plastic autosampler vials.

3.3. Analysis

1. Place the sample extracts on the autosampler in the following order (see Note 2):

   Unextracted control.

   Calibration standards, in order of lowest to highest concentration.

   Negative urine (carryover) control.

   Quality controls, lowest to highest.

   Unknown samples and additional quality controls.

2. Set LC-MS/MS method to the following parameters:
   (a) Autosampler parameters: Cool to constant 7°C. Inject 30 µL per sample.
   (b) LC parameters:

   Column temperature: 38°C with a flow rate of 0.27 mL/min.

   Mobile phase program:

   0.0–1.0 min: Hold at 5% MP-B.

   1.0–15.0 min: Linear gradient to 35% MP-B.

   15.0–15.5 min: Linear gradient to 95% MP-B.

   15.5–16.0 min: Hold at 95% MP-B.

   16.0–16.5 min: Linear gradient to 5% MP-B.

   16.5–17.0 min: Hold at 5% MP-B.

   (c) Ion source parameters: Gas temp = 350°C, gas flow = 10 L/min, nebulizer = 30 psi, and capillary voltage = 3,500 V.
   (d) Mass spectrometer parameters: Compound-dependent parameters are detailed in Table 3 (see Note 3).
3. Figure 2 shows the chromatography of a standard containing all analytes and ISs. One quantitating transition and two qualifying transitions are shown for each compound (Fig. 2).

Table 3
Analytical and detection conditions for cocaine, associated analytes, and internal standards

Drug	Retention time (min)	Precursor ion *(m/z)*	Quantifier product ion *(w/z)*	Quant. collision energy (V)	Qualifier product ion #1 *(m/z)*	Qual. #1 collision energy (V)	Mean ratio qualifier MRM #1	Qualifier product ion #2 (M/Z)	Qual. #2 collision energy (V)	Mean ratio qualifier MRM #2
AEME[a]	1.4	182.1	91.1	28	122.1	18	54.4	118.0	20	59.5
m-HOBE	4.9	306.1	168.1	20	150.1	26	6.8	121.0	32	25.1
BE	6.6	290.1	168.1	18	105.0	35	27.0	82.1	35	17.0
BE-d_3	6.6	293.2	171.1	19	105.0	35	30.0	85.1	33	17.0
COC[a]	9.4	304.2	182.1	18	105.0	36	13.5	82.1	32	29.5
COC-d_3	9.4	307.2	185.1	20	105.0	37	14.6	85.1	34	30.0
NC[a]	10.2	290.1	168.1	14	136.1	24	78.0	108.1	33	17.5
CE	11.8	318.2	196.1	19	150.1	26	11.4	82.1	31	43.2
CE-d_8	11.8	326.2	204.2	20	153.1	31	7.6	85.1	35	36.0

[a]Internal standard is D_3-cocaine

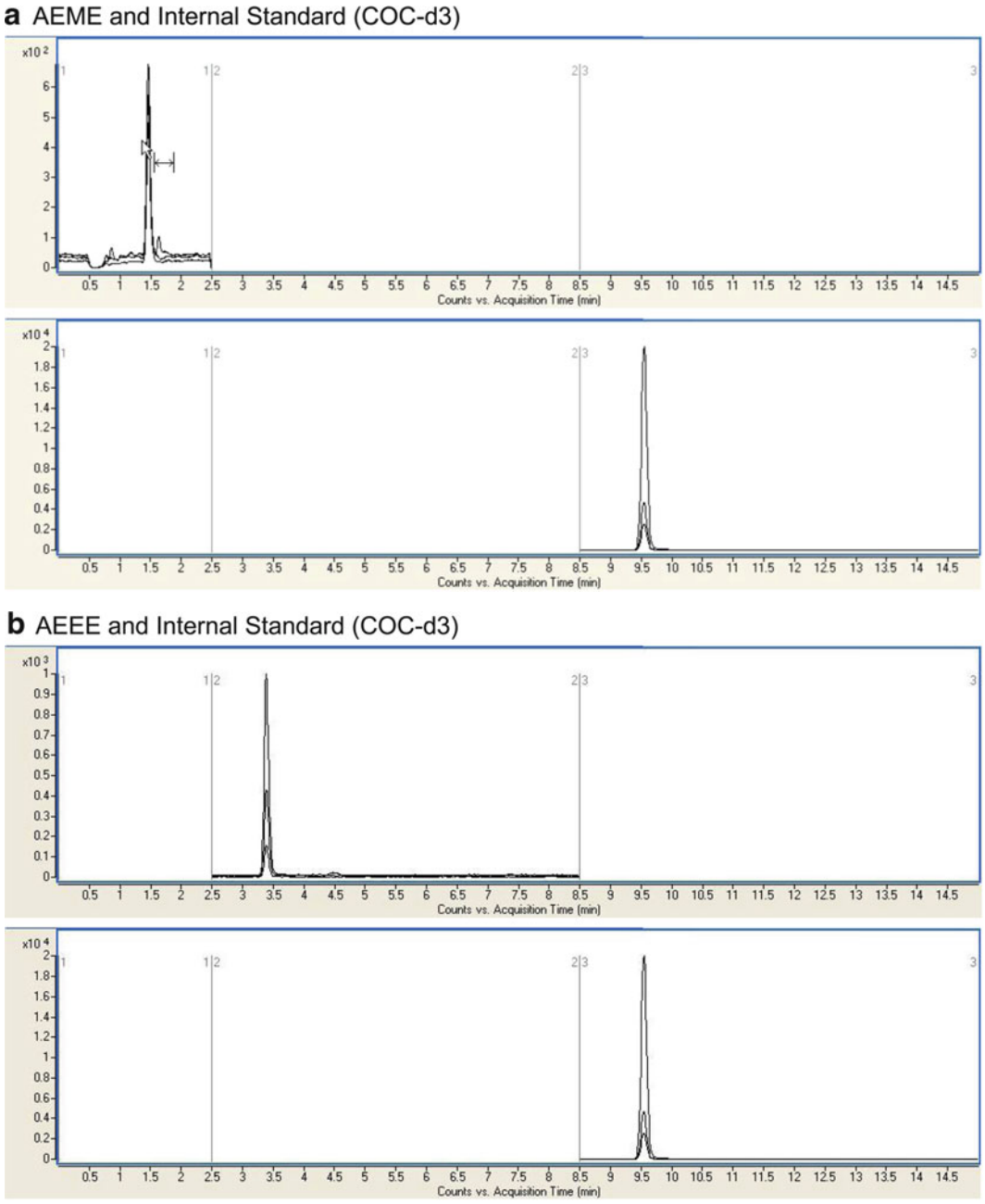
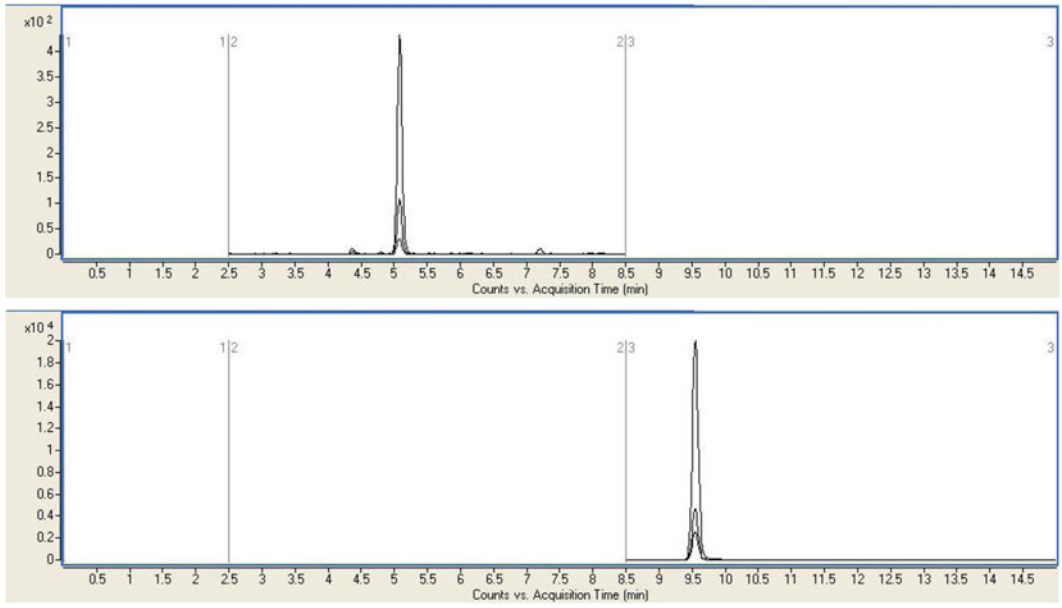


Fig. 2. Representative chromatogram at the limit of quantitation (1.0 ng/mL) for cocaine, associated analytes, and internal standards.

c M-HOBE and Internal Standard (COC-d3)



d BE and Internal Standard (BE-d3)

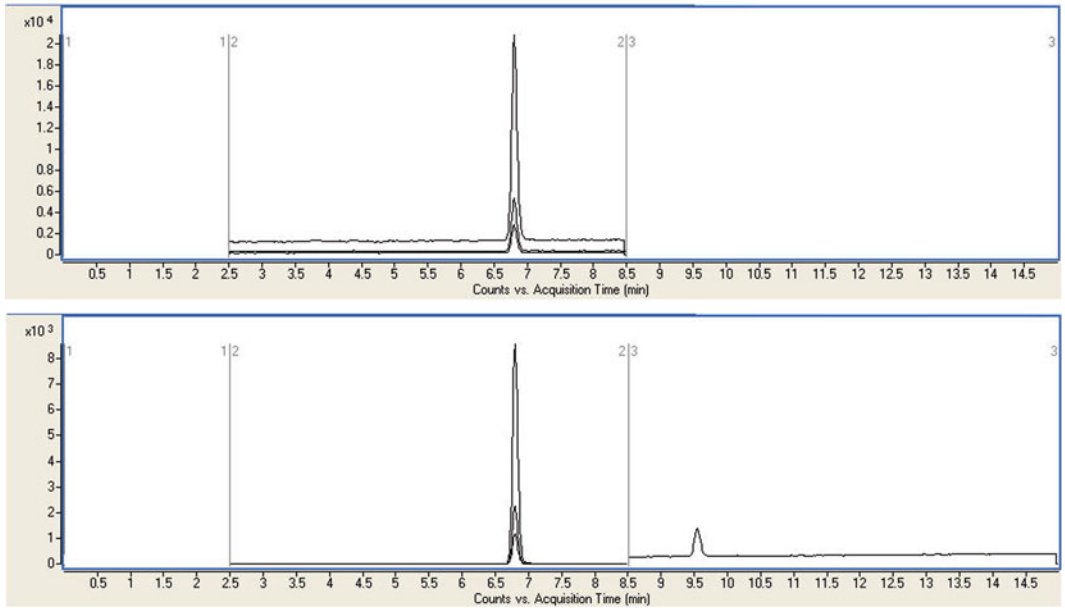
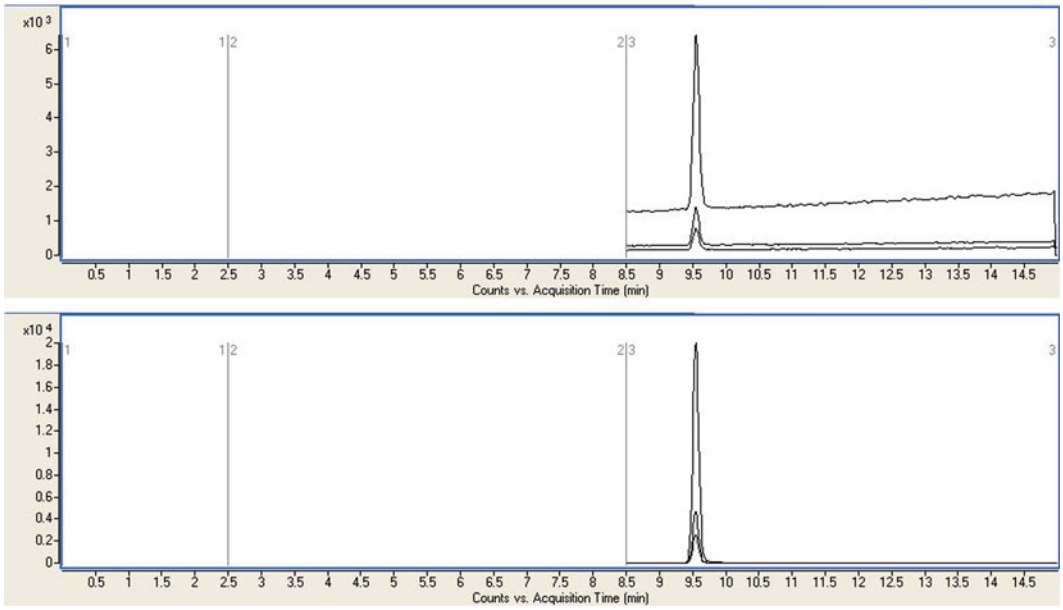


Fig. 2. (continued)

e COC and Internal Standard (COC-d3)



f NC and Internal Standard (COC-d3)

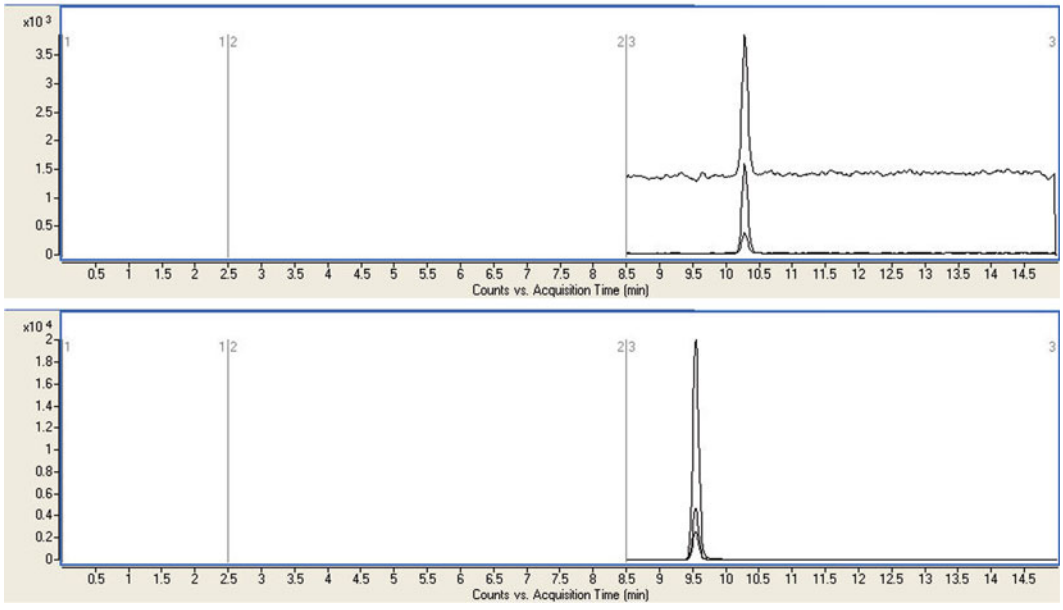


Fig. 2. (continued)

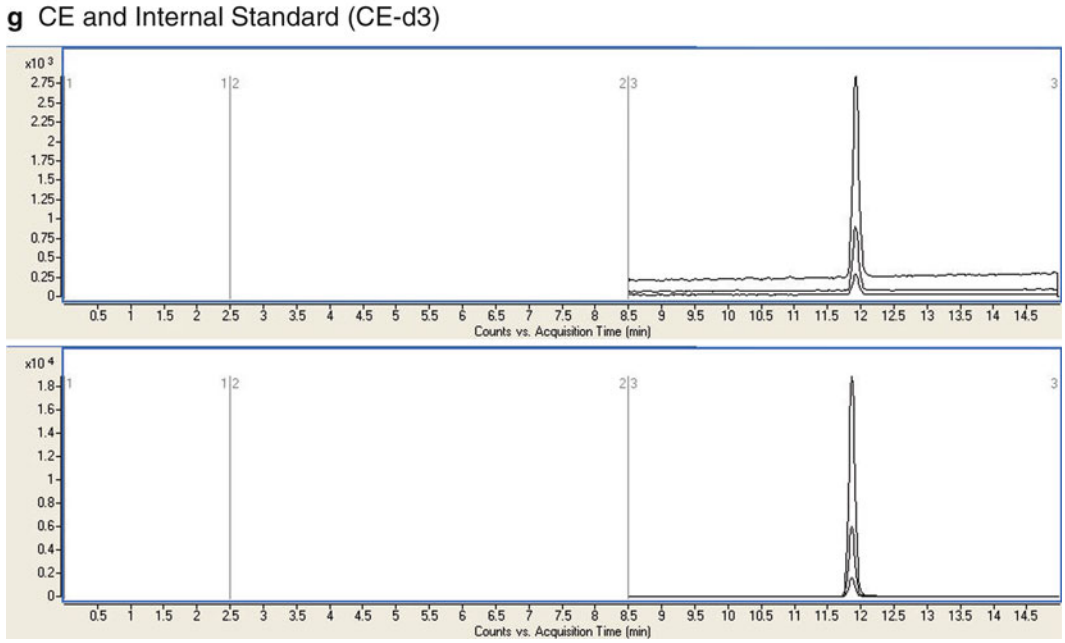


Fig. 2. (continued)

4. Notes

1. For samples expected to have high values, it is recommended to analyze both the undiluted (neat) specimen and a 50× dilution in drug-free urine. Concentrations of BE above the analytical range (i.e., >100 ng/mL) can result in poor chromatography for both the BE and COC peaks. This can be resolved by adequate dilution of the sample.
2. Sample order is at the discretion of the user; the rationale for this order is as follows: the unextracted control confirms instrument performance independently of the success of the extraction. 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 that at least 10% of each clinical run comprises quality controls and calibrators, we run one control after every nine patient samples.
3. Quantifier and qualifier multiple reaction monitoring (MRM) transitions are based off a single precursor ion for each drug. Mass spectrometry parameters will vary between instruments and must be optimized for the specific LC-MS/MS used.

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Chapter 9

Quantitation of Amphetamine-Type Stimulants by LC-MS/MS

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Abstract

Amphetamines or amphetamine-type stimulants (ATSs) refer to a group of pharmacological and toxicological agents that have a common phenethylamine structural backbone and typically impart effects that include, but are not limited to, vasoconstriction, anorexia, central nervous system stimulation, and/or hallucinations. While differences in side chain chemistry can impart different pharmacological or toxicological effects, for some compounds, e.g., MDMA (Ecstasy), alterations of the phenyl part of the molecule impart other significant effects. ATSs are used both therapeutically and recreationally, with significant abuse and addiction potential. Therapeutically, these agents are mainly used to treat hyperactivity disorders or aid in weight loss. Toxicological effects include hypertension, arrhythmia, excitability, aggressiveness, psychoses, coma, and death.

Traditional analytical methods to analyze amphetamines include gas chromatography–mass spectrometry where derivatization is often required to facilitate analysis. Besides sample preparation issues, it has been demonstrated that injection port chemistry in the GC can lead to misleading results with some members of the amphetamine class. To circumvent these issues, liquid chromatography-mass spectrometry (LC-MS/MS) offers the promise of a simpler sample preparation procedure and fewer analytical concerns. This chapter describes an LC-MS/MS technique for the analysis of 14 ATSs in blood, serum/plasma, and urine. The method is quantitative and has reporting limits in the low ng/mL range. Electrospray ionization is used in the positive ion mode. Two transitions for each compound are monitored along with ion ratios.

Key words: Amphetamines, ATS, Blood, Serum/plasma, Urine, LC-MS/MS, Positive ESI, Quantitative

1. Introduction

Amphetamine-type stimulants (ATSs) are a broad class of substances that, structurally, have a phenethylamine backbone. Changes to the side chain impart greater or lesser effects to the molecule in respect to pharmacological and toxicological

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actions, although not necessarily uniformly (1). Additionally, changes to the phenyl moiety, e.g., addition of a dioxy bridge, can significantly affect the pharmacological and toxicological actions of the substance compared to the classic phenethylamine structure (2).

These compounds have been used therapeutically for many years in the treatment of hyperactivity disorders and to facilitate weight loss. Additionally, other therapeutic uses of some of the ATSs exist, e.g., selegiline for Parkinson's disease and dementia (3) or pseudoephedrine for nasal congestion. Not all of the amphetamines have therapeutic uses. In this respect, both licit and illicit amphetamines are subject to abuse, which can lead to addiction. Based on their abuse and addiction properties as well as a lack of, or limited, medical use, many of the amphetamines are classified in the United States as schedule I and II controlled substances (4).

Common toxicological effects of amphetamines include hypertension, arrhythmia, excitability, aggressiveness, psychoses, cardiovascular accidents, coma, and death. Additionally, amphetamines can cause hallucinations. It should be noted, however, that not all adverse effects are observed with all compounds in this class of agents, and some adverse effects, e.g., "formication," may be limited to certain amphetamines (5, 6).

The analysis of ATSs is long-standing in toxicology. Some of the earlier methods of analysis, e.g., spectrophotometry, have given way to more specific methods based on mass spectrometry. However, due to their lack of characteristic or significant fragmentation, many of the amphetamines are a challenge even through mass spectrometric means of analysis. In gas chromatography–mass spectrometry (GC–MS), many of the amphetamines require derivatization to facilitate analysis (7). Further, in addition to sample preparation issues, it has been demonstrated that injection port chemistry in GC can lead to misleading results with some members of the amphetamine class (8). To circumvent these issues, liquid chromatography-mass spectrometry (LC-MS/MS) offers the promise of a simpler sample preparation procedure and fewer analytical concerns. For example, no derivatization is needed in LC-MS/MS and given the softer ionization process, more distinctive fragmentation can be taken advantage of to aid in the qualitative and quantitative aspects of the analysis. Described herein is an LC-MS/MS technique for the analysis of 14 amphetamines in blood, serum/plasma, and urine using isotope dilution for the majority of compounds. The method is quantitative and has reporting limits in the low ng/mL range. Electrospray ionization is used in the positive ion mode. Two transitions for each compound are monitored along with ion ratios (see Note 1).

2. Materials

2.1. Reagents

1. 10% Trichloroacetic acid in water.
2. Mobile Phase A (MP-A)—0.1% formic acid in deionized water.
3. Mobile Phase B (MP-B)—0.1% formic acid in methanol.
4. Blank (drug-free) urine, serum/plasma, blood.

2.2. Standards

1. *Stock solutions.* The following are available as 1 mg/mL methanolic stocks (Cerilliant, Round Rock, TX): ephedrine, methylephedrine, pseudoephedrine, phenylpropanolamine, amphetamine, phentermine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), selegiline, d_3-ephedrine, d_3-phenylpropanolamine, d_3-amphetamine, d_5-methamphetamine, d_5-MDA, d_5-MDMA, d_5-MDEA. Norpseudoephedrine, d_8-selegiline, and d_3-pseudoephedrine are available as 0.1 mg/mL methanolic stocks (Cerilliant, Round Rock, TX). Phendimetrazine (Alltech Associates, Lancaster, PA) and phenmetrazine (USP, Rockville, MD) are available as powders; to create the stock standards, dissolve the powders in methanol to a concentration of 1 mg/mL.
2. *Mixed amphetamines panel substock standard A.* Add 5 mL of deionized water to a 10 mL volumetric flask. Add 10 µL of concentrated hydrochloric acid and mix well. Quantitatively add the stock standards as follows: 100 µL each ephedrine, methylephedrine, pseudoephedrine, phenylpropanolamine, amphetamine, phentermine, methamphetamine, MDA, MDMA, phendimetrazine, phenmetrazine; 200 µL MDEA; 50 µL selegiline; 1,000 µL norpseudoephedrine. Dilute to volume with deionized water; cap and mix by inversion. Store between 2 and 8°C in an amber bottle with Teflon®-lined cap. Final concentration for all compounds is 10 µg/mL except MDEA (20 µg/mL) and selegiline (5 µg/mL).
3. *Mixed amphetamines panel substock standard B.* Accurately transfer 1 mL of substock A to a 10 mL volumetric flask. Dilute to volume with deionized water; cap and mix by inversion. Store between 2 and 8°C in an amber bottle with Teflon®-lined cap. Final concentration for all compounds is 1 µg/mL except MDEA (2 µg/mL) and selegiline (0.5 µg/mL).
4. *Amphetamines panel working calibrators.* Transfer 0.2 mL aliquots of blank serum/plasma, blood, or urine, depending on the matrix of interest, to 12×75 mm test tubes. Add the

Table 1
Working calibrator preparation

Standard	1	2	3	4	5	6	7
Amount substock A or B	20 µL A	10 µL A	4 µL A	10 µL B	4 µL B	2 µL B	1 µL B
Component	Concentration (ng/mL)						
MDEA	2,000	1,000	400	100	40	20	10
Selegiline	500	250	100	25	10	5	2.5
All other compounds	1,000	500	200	50	20	10	5

appropriate volume of substock A or B as designated in Table 1. Vortex briefly to mix. These working calibrators should be made fresh with each analytical run.

5. *Mixed amphetamine working internal standard*. In a 1,000 mL volumetric flask, add 500 mL deionized water and 100 µL of concentrated HCl; mix well (see Note 2). Quantitatively add the following stock standards to the flask: 0.5 mL each d_3-ephedrine, d_3-phenylpropanolamine, d_5-amphetamine, d_5-methamphetamine, d_5-MDA, d_5-MDMA, d_5-MDEA; 5 mL each d_3-pseudoephedrine and d_8-selegiline. Dilute to volume with deionized water and mix by inversion. Store between 2 and 8°C in an amber bottle with Teflon®-lined cap. Final concentration for all labeled compounds is 0.5 µg/mL.

2.3. Controls (see Note 3)

1. *Mixed amphetamines panel stock QC solution*. Into a 50 mL volumetric flask, transfer stock standards (1 or 0.1 mg/mL) as follows: 0.5 mL each ephedrine, methylephedrine, pseudoephedrine, phenylpropanolamine, amphetamine, phentermine, methamphetamine, MDA, MDMA, phendimetrazine, phenmetrazine; norpseudoephedrine; 1 mL MDEA; 0.25 mL selegiline. Dilute to volume with deionized water. Store between 2 and 8°C in an amber bottle with Teflon®-lined cap. Final concentration for all compounds is 10 µg/mL except MDEA (20 µg/mL) and selegiline (5 µg/mL).
2. *Working controls*. To a 100 mL volumetric flask containing 50 mL blank serum/plasma, blood, or urine, depending on the matrix of interest, add the following amounts of QC stock solution: low QC, 300 µL; mid QC, 3.75 mL; high QC, 7.5 mL. Stable for 1 year when stored frozen (below –10°C) (see Note 4). Final concentrations are as follows: low QC, 30 ng/mL except MDEA (60 ng/mL) and selegiline (15 ng/mL); mid QC, 375 ng/mL except MDEA (750 ng/mL) and

selegiline (187.5 ng/mL); high QC, 750 ng/mL except MDEA (1,500 ng/mL) and selegiline (375 ng/mL).

3. *Dilution QC*. To confirm the accuracy of sample dilution, prepare a dilution QC for each run as needed. The dilution QC is made by adding 25 µL of the high QC to 225 µL of the appropriate blank matrix. Mix well and transfer 0.2 mL to a new tube for analysis.

2.4. Equipment

The instrument employed is a Waters Micromass Quattro Premier with a Waters Acquity Ultra Performance LC equipped with Mass Lynx software. The pre-column is a VanGuard HSS T3 1.8 µm, 2.1 × 5 mm, or equivalent. The analytical column is an Acquity UPLC HSS T3 1.8 µm, 2.1 × 50 mm, or equivalent.

3. Methods

Note—Due to the use of chemicals and material of biological origin, procedures consistent with a laboratory's Chemical Hygiene and Bloodborne Pathogens standard operating procedures must be followed.

3.1. Procedure

1. Transfer 0.2 mL blank serum/plasma, blood and/or urine, standards, controls, and patient specimens to appropriately labeled 12 × 75 mm tubes (see Note 5). Multi-point calibrators are run in the beginning of an analytical run with controls interspersed throughout the run according to the QC standards established by a laboratory. The last sample of a run should be a low QC.
2. Add 100 µL of mixed amphetamines panel working internal standard to each tube; vortex to mix.
3. Add 200 µL of 10% trichloroacetic acid. Vortex for 30 s.
4. Centrifuge all tubes for approximately 5 min at an approximate rcf = 800 × *g* (see Note 6).
5. Transfer 200 µL of supernatant by pipette to autosampler vials and seal with pre-slit Teflon-lined snap caps. Extracts are now ready for LC-MS/MS analysis.
6. Inject 5 µL volume onto the instrument.

3.2. Analysis

1. Instrument parameters are listed in Table 2. The target column temperature is 40°C. The flow rate is 0.4 mL/min.
2. Gradient parameters are as follows:

   Initial: 5% MP-B.

   0–3 min: Linear gradient to 10% MP-B.

Table 2
Instrumental parameters

Parameter	Start mass
MS scan	73

Source (ES+)	Settings
Capillary (kV)	0.50
Cone (V)	12.00
Extractor (V)	3.00
RF (V)	0.3
Source temperature (°C)	100
Desolvation temperature (°C)	350
Cone gas flow (L/h)	54
Desolvation gas flow (L/h)	776

Analyzer	Settings
Collision gas flow (mL/min)	0.20
LM 1 resolution	13.0
HM 1 resolution	13.0
Ion energy 1	0.50
Entrance	0
Collision	20
Exit	1
LM 2 resolution	13.0
HM 2 resolution	13.0
Ion energy 2	1.0
Multiplier (V)	650
Syringe pump flow (μL/min)	25.0

3–5 min: Linear gradient to 20% MP-B.

5–6.7 min: Linear gradient to 95% MP-B.

6.7–6.9 min: Hold at 95% MP-B.

6.9–7 min: Linear gradient to 5% MP-B.

7–8 min: Hold at 5% MP-B.

3. The mass transitions and compound-specific parameters are listed for internal standards and analytes in Fig. 1 and Table 3.

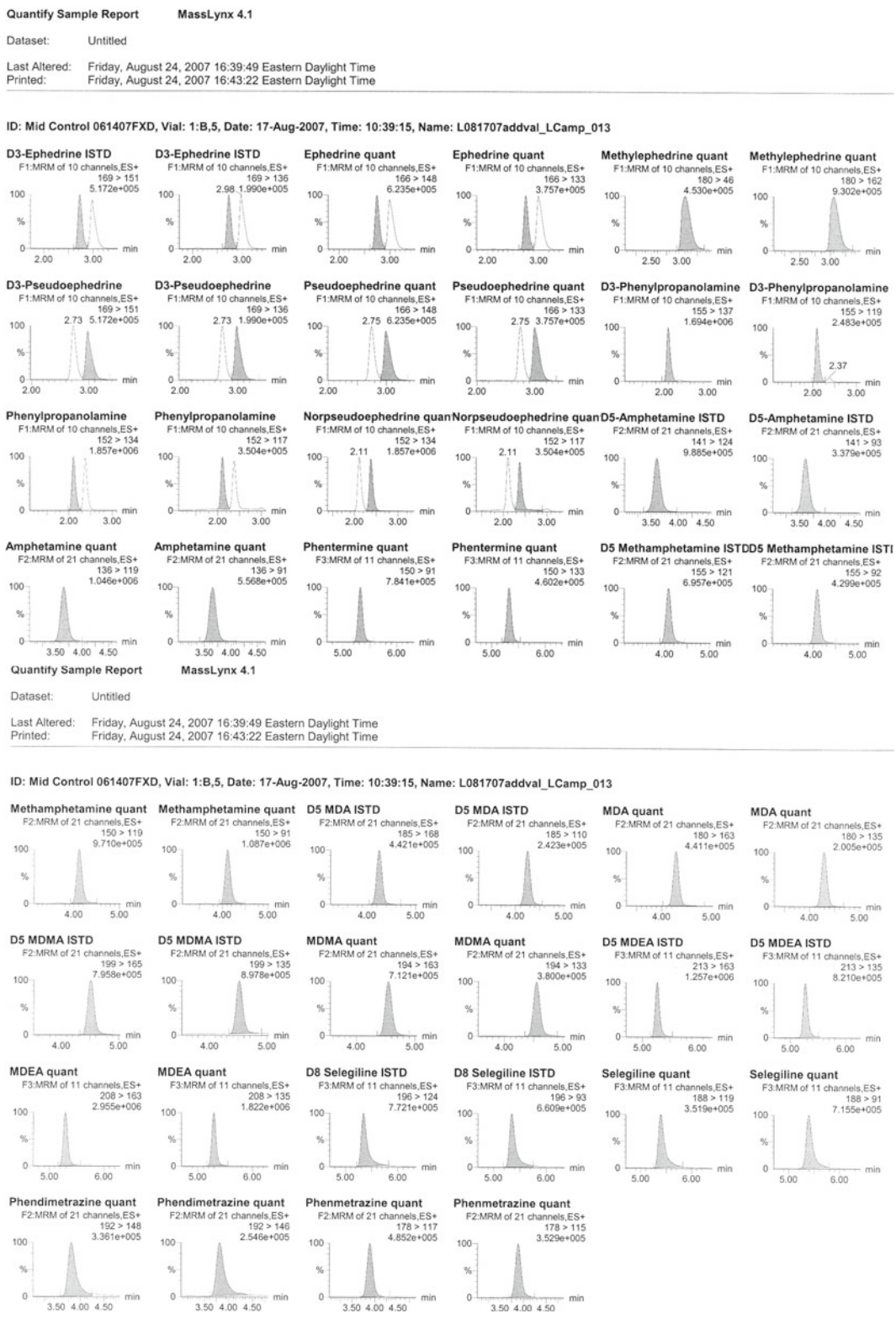

Fig. 1. Typical MRM LC-MS/MS data for amphetamines analysis.

Table 3
Compound parameters

Analyte	Quantifier MRM	Qualifier MRM	Cone (V)	Collision (eV)	Retention time (min)
D_3-phenylpropanolamine	155>137	155>119	15	15	2.09
Phenylpropanolamine	152>117	152>134	15	15	2.10
Norpseudoephedrine	152>134	152>117	15	15	2.37
D_3-ephedrine	169>151	169>136	15	22	2.73
Ephedrine	166>148	166>133	15	22	2.75
Methylephedrine	180>146	180>162	40 (quant) 20 (qual)	20	3.07
D_3-pseudoephedrine	169>151	169>136	15	22	2.98
Pseudoephedrine	166>148	166>133	15	22	2.99
D_5-MDA	185>168	185>110	15	18	4.25
MDA	180>163	180>135	15	18	4.29
D_5-amphetamine	141>124	141>93	15	10	3.60
Amphetamine	136>119	136>91	15	10	3.65
Phentermine	150>91	150>133	15	15 (quant) 8 (qual)	5.32

D_5-methamphetamine	155 > 121	155 > 92	18	12	4.09
Methamphetamine	150 > 119	150 > 91	18	12	4.12
Phendimetrazine	192 > 148	192 > 146	30 (quant) 28 (qual)	25	3.86
Phenmetrazine	178 > 117	178 > 115	30	22 (quant) 25 (qual)	3.90
D_5-MDMA	199 > 165	199 > 135	20	20	4.52
MDMA	194 > 163	194 > 133	20	20	4.55
D_5-MDEA	213 > 163	213 > 135	20	20	5.27
MDEA	208 > 163	208 > 135	20	20	5.30
D_8-selegiline	196 > 124	196 > 93	22	15	5.36
Selegiline	188 > 119	188 > 91	22	15	5.41

Each compound is listed below the internal standard used to quantitate it

4. Each analyte must meet retention time parameters as established by a laboratory, e.g., (±2% of calibrators). Ion ratio characteristics (e.g., ±30% of calibrators) must also be met. Quantitation is based on response of the analyte of interest to the internal standard response compared to the same ratios that generate the calibration curve.

4. Notes

1. Acceptable ion ratios are ±30% to accommodate varying ion responses. If a laboratory so chooses, individual ion ratios for each analyte can be established with individual acceptance criteria.
2. Hydrochloric acid is added to stabilize some analytes as HCl salts.
3. Stock standards used for QC solutions are prepared from a separate lot, a separate source, or a separate weighing of the material.
4. Approximately 0.3 mL aliquots are stored in micro conical tubes. Stability experiments have demonstrated stability of the analytes after three freeze–thaw experiments.
5. Urine samples are run with a 1 + 9 dilution to accommodate the relatively high concentrations found in urine. Samples can always be run undiluted if necessary.
6. If sample appears turbid after this step, an additional centrifugation should be done using a fixed-angle rotor in microfuge tubes.

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Chapter 10

Detection of Prohibited Substances by Liquid Chromatography Tandem Mass Spectrometry for Sports Doping Control

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Abstract

Drug testing for sports doping control programs is extensive and includes numerous classes of banned compounds including anabolic androgenic steroids, β2-agonists, hormone antagonists and modulators, diuretics, various peptide hormones, and growth factors. During competition, additional compounds may also be prohibited such as stimulants, narcotics, cannabinoids, glucocorticosteroids, and beta-blockers depending both on the sport and level of competition. Each of these classes of compounds can contain many prohibited substances that must be identified during the testing procedure. Various methods that have been designed to detect a large number of compounds in different drug classes are highly desirable as initial screening tools. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) is widely used by anti-doping testing laboratories for this purpose and several rapid methods have been described to simultaneously detect different classes of compounds. Here, we describe a simple urine sample cleanup procedure that can be used to detect numerous anabolic androgenic steroids, β2-agonists, hormone antagonists and modulators, glucocorticosteroids, and beta-blockers by LC-MS/MS.

Key words: Liquid chromatography, Mass spectrometry, Sports doping, Urine drug testing, World Anti-Doping Agency, Anabolic agents, Beta-blockers, β2-Agonists, Glucocorticoids, Anti-estrogenic agents

1. Introduction

The World Anti-Doping Agency (WADA) was formed in 1999 to combat the use of performance-enhancing substances in sports. Signatories to the World Anti-Doping Program such as the International Olympic Committee, International Federations, and National Anti-Doping Organizations are required to follow all provisions of the World Anti-Doping Program including the *Code* (specific anti-doping rules and principles), which contains the

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Table 1
The WADA 2010 list of prohibited substances

1. Anabolic androgenic steroids Exogenous Endogenous Other anabolic agents
2. Peptide hormones, growth factors and related substances
3. β2-Agonists
4. Hormone and metabolic modulators Aromatase inhibitors Selective estrogen receptor modulators Other anti-estrogenic substances Agents modifying myostatin function Metabolic modulators
5. Diuretics and other masking agents
6. Stimulants (in competition only)
7. Narcotics (in competition only)
8. Cannabinoids (in competition only)
9. Glucocorticosteroids (in competition only)
10. Alcohol and beta-blockers (in competition only, some sports)

prohibited list of substances that are banned in- and out-of-competition (1). The 33 WADA-accredited doping control laboratories throughout the world (2) are required to develop active anti-doping research programs and to develop analytical methods to detect the substances on the prohibited list (Table 1).

For sport doping control, many of the methods are designed to test urine specimens. A large volume of urine is easy to collect and less invasive than the collection of a blood sample. Furthermore, most target compounds remain in the bloodstream for only short periods of time and are rapidly metabolized and excreted into the urine. When analyzing urine, it is possible to detect both parent drug and metabolites for several hours after the drug has been cleared from the circulation.

Given the large number of compounds that are monitored in sports doping control programs no single screening method can detect all the relevant compounds. Therefore, liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods are typically used in conjunction with other techniques such as gas

chromatography/mass spectrometry (GC-MS), immunoassays and electrophoretic techniques. LC has advantages over GC separation methods such as the ability to detect thermolabile, volatile, and polar compounds using simple sample clean-up procedures, and typically does not require sample derivatization for good chromatographic separation. Although both GC-MS and LC-MS can be used to detect the anabolic androgenic steroid stanozolol, LC-MS methods have a lower detection limit. Other anabolic androgenic steroids such as tetrahydrogestrinone are routinely detected by LC-MS because the pertrimethylsilyl derivative of tetrahydrogestrinone is unstable and undetectable by GC-MS analysis (3).

For doping control purposes, screening methods are designed to maximize the detection of as many compounds as possible at the expense of optimum conditions for individual compounds. Tandem MS systems (MS/MS) have gained widespread use because this technique can be used to isolate ions in order to determine their relationship with other ions (either generated at the same time or during subsequent fragmentation), thereby providing additional structural information. The detection of target compounds by LC-MS/MS is achieved by comparing the retention time and relative intensities of multiple reaction monitoring (MRM; also called selected precursor/product ion pairs) from a sample to those obtained from the analysis of a reference compound. The likelihood that a target compound and an interfering substance will have the same precursor/product ion pair is considerably lower than the likelihood that the two spectra will contain the same fragment ion due to the electrospray ionization method used in LC-MS. Electrospray ionization is a "soft" ionization producing mainly protonated or deprotonated molecular ions. The use of MRM makes LC-MS/MS data relatively easy to interpret and amenable to software automation.

LC-MS/MS methods are routinely used in doping control programs to detect several classes of compounds on the WADA prohibited list. For example, LC-MS/MS methods can be used to detect 17-alkyl-substituted anabolic steroids (4) and a combination of anabolic steroids and anti-estrogenic agents after a simple derivatization step (5). Rapid LC-MS/MS screening methods have also been developed to simultaneously detect beta-blockers and diuretics (6), synthetic anabolic steroids, glucocorticoids, anti-estrogenic agents and some stimulants (7), and a combination of diuretics, beta-blockers, anabolic steroids, and selected stimulants (8). In this report we describe a simple urine sample cleanup procedure that can be used to detect numerous anabolic androgenic steroids, β2-agonists, hormone antagonists and modulators, glucocorticosteroids, and beta-blockers by LC-MS/MS.

2. Materials

1. 0.2 M phosphate buffer: Weigh out 0.942 g of KH_2PO_4 and 2.96 g of $Na_2HPO_4 \cdot 7H_2O$, and transfer to a 0.5 L glass-stoppered bottle. Add 100 mL of deionized water. Add a magnetic stirring bar to the bottle and stir until solids dissolve completely. Adjust to pH 7.0 by dropwise addition of 12 N NaOH.
2. 100 mM ammonium acetate buffer: To a 1 L volumetric flask, add 800 mL of deionized water, a magnetic stir rod, and 7.708 g of ammonium acetate. Stir until solids dissolve completely. Adjust to pH 3.5 by dropwise addition of glacial acetic acid. Bring to 1 L final volume with deionized water. Stopper and invert several times to mix thoroughly.
3. 5 mM ammonium acetate buffer: To a 1 L volumetric flask, add 850 mL of deionized water, a magnetic stir rod, and 50 mL of 100 mM ammonium acetate buffer. Stir thoroughly. Adjust the pH to 3.5 by dropwise addition of 1 M ammonium hydroxide or glacial acetic acid. Bring to a 1 L final volume with deionized water, stopper, and invert several times to mix thoroughly.
4. Reconstitution solvent (35:65 acetonitrile:5 mM ammonium acetate buffer): Transfer 25 mL of 100 mM ammonium acetate buffer to a 500 mL volumetric flask and add 300 mL of deionized water. Adjust the pH to 3.5 by dropwise addition of glacial acetic acid or 1 M ammonium hydroxide. Add deionized water to bring the volume to 500 mL. Stopper and invert several times to mix thoroughly. Transfer to a 1 L container and add 270 mL of HPLC-grade acetonitrile.
5. Enzyme: β-glucuronidase suspension from *E. coli* K 12 source (EC 3.2.1.31). At 37°C the solution should have at least 140 U/mL of activity.
6. Solid buffer: Mix sodium bicarbonate and sodium carbonate in a 1:1 (w:w) ratio.
7. Internal standard solution: 5.0 μg/mL 17α-methyltestosterone and 0.5 μg/mL d3-3′-hydroxystanozolol, prepared in methanol.
8. Extracted calibrator: Prepared in drug-free urine by adding anastrozole, betamethasone, budesonide, ciclesonide, exemestane, 16α-hydroxyprednisolone, prednisolone, testolactone, tetrahydrogestrionone, and triamcinolone, each at 30 ng/mL; clenbuterol, epitrenbolone, 3′-hydroxystanozolol, 16β-hydroxystanozolol, and N-desmethyltamoxifen, each at 10 ng/mL; oxandrolone, and a formebolone metabolite

(2-hydroxy-17α-methylandrosta-1,4-diene-11α,17β,diol-3-one), each at 20 ng/mL; gestrinone at 40 ng/mL; and salmeterol at 50 ng/mL.

9. Positive control urine: Prepared in drug-free urine by adding letrozole metabolite (bis(cyanophenyl)methanol), 6α-methylprednisolone, prednisone, triamcinolone acetonide, and triamcinolone hexacetonide, each at 30 ng/mL; carvedilol at 10 ng/mL; clenbuterol, epitrenbolone, 3′-hydroxystanozolol, and 16β-hydroxystanozolol, each at 2 ng/mL; gestrinone and tetrahydrogestrinone, each at 4 ng/mL; and finasteride and formoterol, each at 50 ng/mL.
10. Unextracted calibrator for anti-estrogenic agents: Prepared in reconstitution solvent by adding aminoglutethimide, anastrozole, letrozole metabolite (bis(cyanophenyl)methanol), clomiphene, dutasteride, exemestane, fadrozole, finasteride, formestane, fulvestrant, 4-hydroxytamoxifen, N-desmethyltamoxifen, raloxifene, tamoxifen, testolactone, and toremifene, each at 2.5 μg/mL; and 17α-methyltestosterone at 4.0 μg/mL. On the day of use, transfer 50 μL to a chromatographic vial and cap.
11. Unextracted calibrator for glucocorticoids: Prepared in methanol by adding prednisone, prednisolone, cortisone, hydrocortisone, 6α-methylprednisolone, fluocortolone, fludrocortisone, dexamethasone, triamcinolone, beclomethasone, flumethasone, desonide, budesonide, flunisolide, fluticasone propionate, and carvedilol, each at 1.5 μg/mL; 17α-methyltestosterone at 4 μg/mL; and d3-3′-hydroxystanozolol at 0.4 μg/mL. On the day of use, transfer 50 μL to a chromatographic vial, evaporate the solvent completely under a nitrogen stream at ambient temperature, and add reconstitution solvent to the original volume (see Note 1). Mix the solution thoroughly.
12. Calibrators for beta-blockers and β2-agonists: Carvedilol is present in the unextracted calibrator for beta-blockers at 10 ng/mL and the positive control urine at 1.5 μg/mL; formoterol is present in the positive control urine and salmeterol is present in the extracted calibrator for β2-agonists, both at 50 ng/mL.
13. HPLC column for anabolic agents, glucocorticoids, beta-blockers, and β2-agonists: Reversed phase C12, 4 μm particle size, 50 × 2 mm (Phenomenex).
14. HPLC column for anti-estrogenic agents: Reversed phase C8, 5 μm particle size, 150 × 2 mm (Phenomenex).
15. LC-MS/MS: Gradient and isocratic HPLC, electrospray interface, and MS/MS data acquisition with MRM (also called selected precursor/product ion pairs). The specific instruments used are listed in Note 7.

3. Methods

3.1. Sample Preparation

1. Place 2.5 mL of each urine sample into separate 10 mL conical glass tubes. With every batch of samples, add 2.5 mL of calibrator, negative control urine, and positive control urine into separate glass tubes.
2. Pour enough phosphate buffer into a beaker to allow 1 mL to be added to each sample being prepared for analysis. Bring the buffer to a boil, immediately remove from heat and cool to ambient temperature (see Note 2).
3. Add 40 μL of internal standard solution, followed by 1 mL of phosphate buffer and then 50 μL of β-glucuronidase enzyme (see Note 3). Mix thoroughly.
4. Incubate for 1 h at 50°C.
5. Add solid buffer to each tube to adjust the pH to approximately 9.5. Cap each tube and mix thoroughly.
6. Add 1/4 teaspoon (~1.7 g) of anhydrous sodium sulfate to each glass tube (see Note 4).
7. Add 3 mL of pentane and then 3 mL of diethylether to each tube, cap and mix thoroughly on a mechanical shaker for 10 min (see Note 5).
8. Centrifuge each tube at 655 × *g* for 10 min.
9. Transfer the organic layer from each tube to a new conical glass tube (see Note 6).
10. Completely evaporate the solvent under a nitrogen stream.
11. Add 50 μL of reconstitution solvent to each tube, mix thoroughly, and then transfer each sample to a chromatography vial and cap.

3.2. LC-MS/MS Analysis

1. Fill the mobile phase reservoirs A and B with 5 mM ammonium acetate and acetonitrile, respectively.
2. Perform anabolic agent, β2-agonist, beta-blocker, and glucocorticoid acquisition for all samples, calibrators, and negative and positive control urines using the reversed phase C12 HPLC column at a programmed flow rate of 0.2 mL per minute for 2.65 min, then a flow rate of 0.4 mL per minute from 2.65 to 4.0 min.
3. For this program, the mobile phase gradient is initially 40% acetonitrile. The 40% acetonitrile is held for 1 min, ramped to 98% acetonitrile during the next 1 min, held at 98% acetonitrile for 0.5 min, returned to 40% acetonitrile in 6 s, and then held there for the next 1.4 min.

Table 2
Retention times and MRMs for internal standards anabolic agents, beta-blockers, β2-agonists, and metabolites in the LC-MS/MS screening procedure

Name	Retention time (min)	MRM 1	MRM 2	MRM 3
Internal standards				
17α-Methyltestosterone	1.32	303/97		
d3-3′-Hydroxystanozolol	2.15	348/97		
Anabolic agents				
Clenbuterol	0.72	277/203	277/132	
Gestrinone	2.40	309/241		
Formebolone metabolite	1.00	347/281		
Methyldienolone	2.27	287/159	287/161	287/135
Methyltrienolone	2.26	285/227	285/159	
17-Epioxandrolone	2.48	307/121	307/229	
16-Hydroxyfurazabol	0.97	347/329	347/157	
16-Hydroxyprostanazol	1.44	329/81		
17-Ketoprostanazol	3.23	313/95		
3′-Hydroxystanozolol	1.34	345/97		
16β-Hydroxystanozolol	2.23	345/81		
Tetrahydrogestrinone	2.61	313/241		
17-Epitrenbolone	2.13	271/199	271/253	
Beta-blockers				
Carvedilol	1.15	407/224		
Mepindolol	0.73	263/116	263/186	
β2-Agonists				
Bambuterol	0.72	368/294	368/72	368/312
Fenoterol	0.66	304/135	304/152	
Formoterol	0.71	345/149		
Salmeterol	1.50	416/232	416/248	
Terbutaline	0.73	226/152	226/125	226/107

4. Retention times and MRMs (precursor/product ion transitions) for the internal standards, anabolic agents and metabolites, beta-blockers, β2-agonists, and glucocorticoids are shown in Tables 2 and 3. Depending on the compound, 1–3 product ions are monitored in MRM mode for the screening procedure.
5. Perform anti-estrogenic agents acquisition for all samples, calibrators, negative and positive control urines using the reversed phase C8 HPLC column and an isocratic mobile phase of acetonitrile and 5 mM ammonium acetate buffer at a 70:30 ratio, with a flow rate of 0.2 mL/min. Retention times and MRMs for the internal standard and anti-estrogenic agents

Table 3
Retention times and MRMs for internal standards, glucocorticoids and glucocorticoid metabolites in the LC-MS/MS screening procedure

Name	Retention time (min)	MRM 1	MRM 2	MRM 3
Internal standards				
17α-Methyltestosterone	1.32	303/97		
d3-3′-Hydroxystanozolol	2.15	348/97		
Glucocorticoids				
Beclomethasone	1.87	409/147		
Budesonide	2.66	431/323		
Ciclesonide	3.34	541/323	541/147	
Ciclesonide metabolite	2.93	471/323	471/147	
Deflazacort	2.18	442/400	442/382	
Deflazacort metabolite	1.08	400/382	400/124	400/147
Desonide	2.03	417/323		
Dexamethasone	1.69	393/373		
Fludrocortisone	1.29	381/239	381/91	
Flumethasone	1.82	411/253		
Flunisolide	2.14	435/321		
Fluocortolone	2.30	377/303		
17-Carboxyfluticasone	2.21	453/293	453/275	453/313
Fluticasone propionate	2.95	501/121		
16-Hydroxyprednisolone	0.95	377/323		
6α-Methylprednisolone	1.55	375/161	375/91	375/339
Prednisolone	1.24	361/147	361/171	361/173
Prednisone	1.31	359/147	359/237	359/171
Triamcinolone	0.97	395/375		
Triamcinolone acetonide	2.04	435/397	435/339	435/213
Triamcinolone hexacetonide	3.14	533/397	533/415	533/513

and metabolites are shown in Table 4. Depending on the compound, 1–3 product ions are monitored in MRM mode for the screening procedure.

3.3. LC-MS/MS Data Review

1. Integrate chromatograms for all MRMs and check all data for proper integration of the internal standard(s). Check for proper integration of all target compounds in the calibrators and positive control urine.
2. Check for consistency of internal standard retention times between calibrator, positive control urine, negative control urine, and samples. Internal standard retention times are expected to be stable and within ±0.3 min of the retention times presented in Tables 2–4. Retention times for target compounds present in calibrators and positive control urine are expected to be within ±0.5 min of the retention times presented in Tables 2–4.

Table 4
Retention times and MRMs for internal standard, anti-estrogenic agents, and metabolites in the LC-MS/MS screening procedure

Name	Retention time (min)	MRM 1	MRM 2	MRM 3
Internal standard				
17α-Methyltestosterone	3.19	303/97		
Anti-estrogenic agents				
Aminoglutethamide	2.13	233/146	233/130	233/188
Anastrozole	2.45	294/225		
Clomiphene	3.21	406/100		
Clomiphene metabolite	2.28	422/100	422/58	
Dutasteride[a]	5.11	529/461		
Exemestane	3.44	297/121	297/93	297/105
17-Dihydroexemestane	2.97	299/135	299/121	
Fadrozole	1.92	224/81	224/82	224/116
Finasteride[a]	2.78	373/305		
Finasteride metabolite[a]	2.68	389/333		
Formestane	3.45	303/125	303/113	
Fulvestrant	4.94	607/159		
Letrozole	2.44	284/242		
Letrozole metabolite	2.61	233/102		
Raloxifene	1.78	474/112		
Tamoxifen	3.73	372/129	372/72	
4-Hydroxytamoxifen	2.34	388/129	388/72	
Methoxytamoxifen	5.27	418/72		
N-Desmethyltamoxifen	3.44	358/129		
Testolactone	2.34	301/121	301/91	
Toremifene	3.27	406/204		

[a]These compounds are specific inhibitors of type II and/or type I 5 α-reductase and were removed from the WADA prohibited list in 2010. They are still monitored by our laboratory because they inhibit the conversion of testosterone to 5α-dihydrotestosterone and can alter steroid profiles

3. The negative control urine must not contain any of the target compounds. The chromatograms should not have any integrated peaks within the expected retention time ranges for any of the monitored diagnostic ions.
4. For anabolic agents, chromatographic peaks for the calibrator and positive urine control corresponding to 3′-hydroxystanozolol, 16β-hydroxystanozolol, gestrinone, epitrenbolone, clenbuterol, and tetrahydrogestrinone must be identifiable for all monitored diagnostic ions, and the ratio of the peak heights of the most abundant ion to that of 17α-methyltestosterone should be in excess of 0.1, 0.03, 1.0, 0.3, 0.2, and 10.0, respectively. Alternatively, the peak heights for the most abundant transitions should be in excess of 20,000, 3,000, 10,000,

50,000, 3,000, and 100,000, respectively (see Note 7). For anabolic agents, only metabolites can be detected in the urine for many of the compounds (see Note 8).

5. For beta-blockers, the carvedilol chromatographic peak height for the positive control should be at least 20,000 and the ratio to internal standard in excess of 2.0 (see Note 7).
6. For β2-agonists, the formoterol chromatographic peak height for the positive control should be at least 80,000 and the ratio to internal standard in excess of 6.0. The salmeterol chromatographic peak height for the extracted calibrator should be at least 60,000 and the ratio to internal standard in excess of 2.0 (see Note 7).
7. For glucocorticoids, the chromatographic peaks for the calibrator corresponding to betamethasone, prednisolone, budesonide, and triamcinolone must be identifiable for all the diagnostic ions and the ratio of the peak heights of the most abundant ion to that of 17α-methyltestosterone should be in excess of 2.0, 0.15, 0.1, and 0.02, respectively. Alternatively the peak heights for the most abundant ion should be in excess of 10,000, 4,000, 4,000, and 1,000, respectively (see Note 7). For the positive control urine, the chromatographic peaks corresponding to prednisone and 6α-methylprednisolone must be identifiable for all the diagnostic ions. For the unextracted calibrator, the chromatographic peaks must be identifiable for all the diagnostic ions for prednisone, prednisolone, methylprednisone, fludrocortisone, betamethasone, dexamethasone, triamicinolone, beclomethasone, flumethasone, desonide, budesonide, flunisolide, and fluticasone. Glucocorticoids are typically detected in the urine as unchanged parent compound (see Note 9).
8. For anti-estrogenic agents, the chromatographic peak of the calibrator for N-desmethyltamoxifen must be identifiable and the ratio of its peak height to that of 17α-methyltestosterone should be in excess of 0.8. Alternatively, the peak height should be in excess of 6,000. For the positive control urine, the chromatographic peaks corresponding to dutasteride, finasteride, and letrozole metabolite must be identifiable for all the diagnostic ions and the ratio of the peak heights of the most abundant ion to that of 17α-methyltestosterone should be in excess of 0.3, 1.0 and 0.3, respectively. Alternatively, the peak heights for the most abundant ion should be in excess of 3,000, 100,000, and 30,000, respectively (see Note 7). For the unextracted calibrator, the chromatographic peaks must be identifiable for all the diagnostic ions for anastrazole, exemestane, fulvestrant, testolactone, toremifene, tamoxifen, 4-hydroxytamoxifen, raloxifen, clomiphene, aminogluthetamide, N-desmethyltamoxifen, dutasteride, finasteride, formestane, letrozole, and letrozole

metabolite. Anti-estrogenic agents can appear in the urine as unchanged parent compound with/without metabolites (see Note 10).

9. For unknown samples, a chromatographic peak in the window for a compound that has only one diagnostic ion that is within ±15 s of the expected retention time of the target compound is indicative of a positive screen. In cases where multiple diagnostic ions are monitored, the presence of a chromatographic peak for each of the ions must be within ±15 s of the expected retention time of the target compound for a positive screen (see Note 11). For compounds with several MRMs, a comparison of the relative peak heights for the ions in the unknown sample and the calibrator/positive control should be performed to increase the discriminating power of the screening procedure. If the relative abundance of the diagnostic ion(s) in the calibrator/positive control is less than 5%, then the relative abundance of the ion in the unknown sample must be ±50%. If the abundance of the diagnostic ion(s) in the known sample is between 5 and 25%, then the absolute abundance in the unknown sample must be ±10%; if between 25 and 50%, then the relative abundance of the unknown sample must be within ±20%; and if >50%, the absolute abundance of the unknown sample must be ±10% to indicate a positive screen.

4. Notes

1. Solvent evaporation can be carried out between ambient temperature and 35°C. Samples can be stored in capped or parafilm-covered tubes after solvent evaporation for a maximum of 24 h before LC-MS/MS analysis.
2. Phosphate buffers are notorious for supporting bacterial growth, so in order to prevent bacterial contamination the buffer is boiled just before use.
3. β-glucuronidase from *E. coli* is recommended for deconjugation of steroids. The same enzyme from *Helix pomatia* should be avoided because it converts 3-beta-5-ene-steroids into 3-oxo-4-ene-steroids. β-glucuronidase from *Helix pomatia* also converts 3-beta-hydroxy-5-alpha-reduced steroids and 3-beta-hydroxy-5-beta-reduced steroids to 3-oxo-5-alpha-reduced steroids and 3-beta-oxo-5-beta-reduced steroids, respectively.
4. Sodium sulfate is used to reduce the volume of the aqueous phase and to enhance extraction by the salting effect.
5. A urine sample will occasionally have the tendency to form a gel or emulsion at this step. In these cases more anhydrous

sodium sulfate should be added to the tube and the sample remixed thoroughly.

6. Only the organic layer should be transferred. If there is an emulsion layer which cannot be eliminated by adding salt, do not transfer the emulsion as it will increase the drying time drastically.
7. The minimum chromatographic peak heights and peak height ratios have been established specifically for the AB SCIEX API 3000, API 4000 and API 4000QTRAP LC-MS/MS instrumentation using static interface settings. LC-MS/MS instrument parameters and interface optimization will dramatically alter both the relative and absolute responses for the diagnostic ions being monitored by this method. If different instrumentation is used to detect these compounds, validation studies will need to be performed to establish criteria for minimum chromatographic peak heights and ratios.
8. Depending on the compound being detected by the LC-MS/MS screening method, the unaltered parent compound, metabolite(s), or both can be detected in the urine specimen. For many anabolic agents, only metabolites can be detected and the concentration of each metabolite can vary significantly. For example, when screening for stanozolol, an anabolic agent, the parent compound is absent from urine whereas the 3′-hydroxy, 16β-hydroxy, and 4β-hydroxy forms of stanozolol are the main metabolites excreted into the urine (9) (Fig. 1a). The 3′-hydroxy and 16β-hydroxy metabolites of stanozolol are monitored in the above-described LC-MS/MS screening method.
9. Classes of compounds such as beta-blockers, β2-agonists, and glucocorticoids are typically detected in urine as the unchanged parent compound. However, it is important to be aware that the same compound can be present in different formulations that have different chromatographic retention times and produce different diagnostic ions that must be separately detected by LC-MS/MS (Table 3). For example, the synthetic glucocorticoid triamcinolone is also available as the acetonide and hexacetonide derivatives (Fig. 1b).
10. Anti-estrogenic agents can be excreted in the urine as unchanged parent compound with or without metabolites. For example, the anti-estrogenic agent exemestane is detected in the urine as unchanged parent compound and as the 17-dihydro metabolite by LC-MS/MS (Fig. 1c).
11. An example showing selected ion chromatographs for clenbuterol in the positive control, negative control, and a positive athletes sample is shown in Fig. 2. Clenbuterol is a polar com-

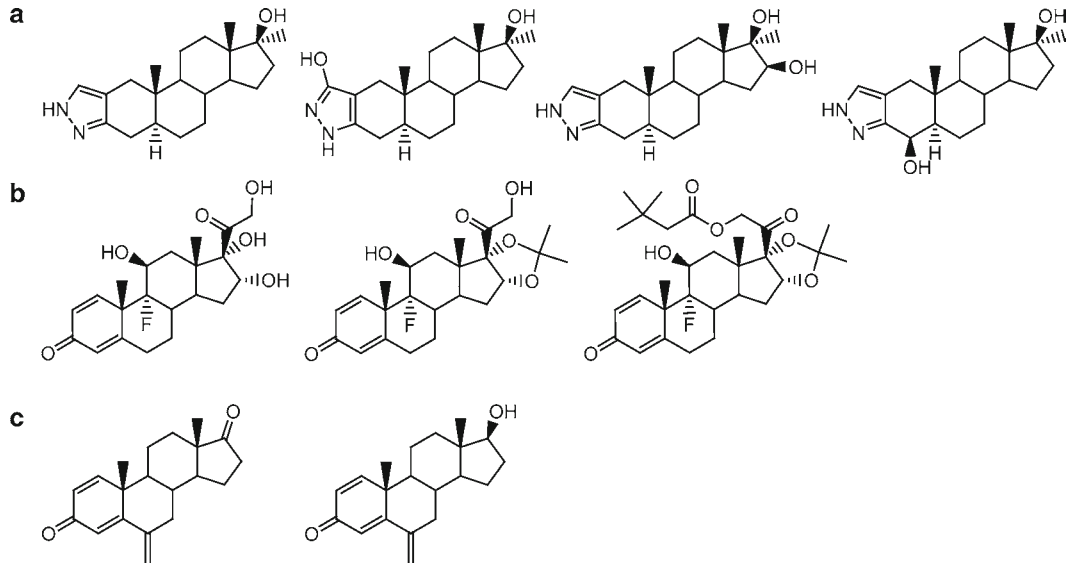

Fig. 1. (**a**) When testing for administration of the anabolic agent stanozolol (*far left panel*) only metabolites such as the 3′-hydroxy, 16β-hydroxy and 4β-hydroxy forms of stanozolol (*left to right* after stanozolol, respectively) can be found in the urine. (**b**) Some compounds such as the glucocorticoid triamcinolone (*left panel*) are also available as the acetonide (*middle panel*) and hexacetonide derivatives (*right panel*). (**c**) The anti-estrogenic agent exemestane can be detected in urine as parent compound (*left panel*) and as the 17-dihydro metabolite (*right panel*).

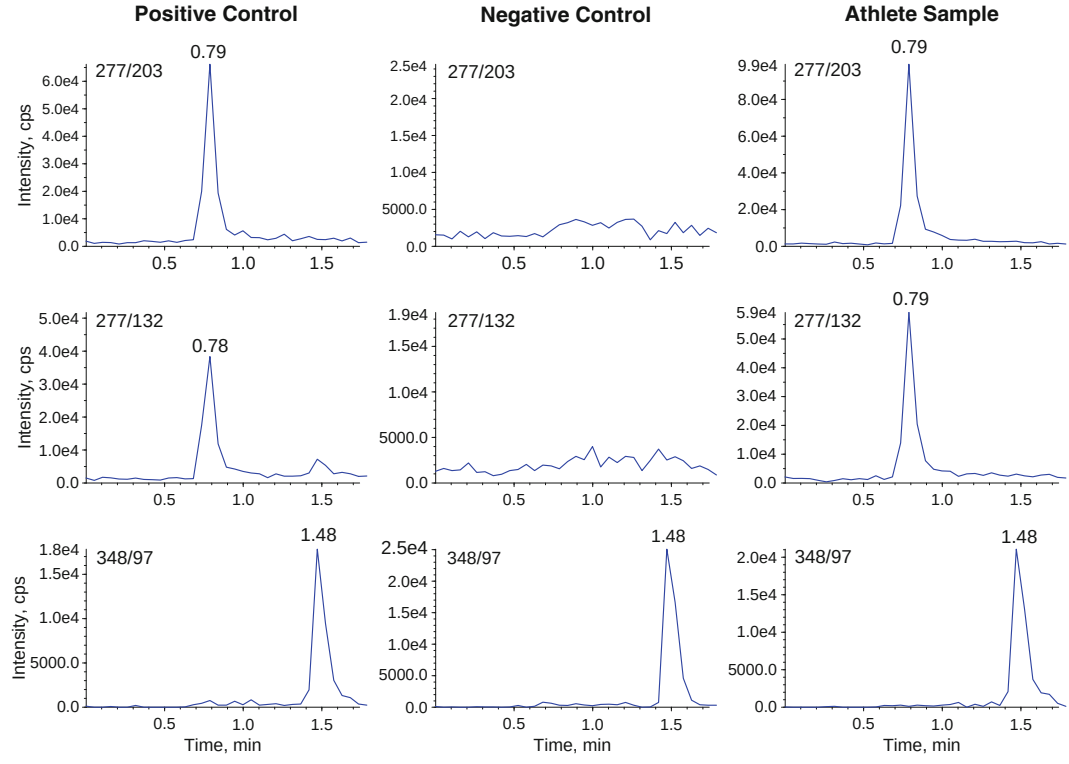

Fig. 2. Selected ion chromatographs for clenbuterol in a positive control urine (*left panels*), negative control urine (*middle panels*), and an athlete's positive urine sample (*right panels*). Clenbuterol MRM *m*/*z* 277 → *m*/*z* 203 and MRM *m*/*z* 277 → *m*/*z* 132 are shown in the *top* and *middle panels*, respectively. The bottom panels are the MRM *m*/*z* 348 → *m*/*z* 97 for the internal standard d3-3′-hydroxystanozolol.

pound that elutes very early (~0.8 min) in the screening method. Many other polar compounds also elute early in this region, and for this reason a second MRM 277/132 was added to the screening method to eliminate false positive results. During the use of the method, we have seen several cases where a peak would appear in the retention time zone for clenbuterol (0.8 min) that would be integrated by the software for MRM 277/203. None of these false positive samples produce a peak with the correct retention time for MRM 277/132 at the appropriate product ion ratio.

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Chapter 11

LC-MS/MS Screen for Xenobiotics and Metabolites

François-Ludovic Sauvage and Pierre Marquet

Abstract

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is now considered as a perfect complement to HPLC-DAD (diode array detection) and gas chromatography (GC)-MS for the general unknown screening of drugs and toxic compounds.

Here we describe a procedure applied routinely in our laboratory for clinical and forensic applications using the QTRAP™ technology.

Key words: Liquid chromatography, Forensic and clinical toxicology, Tandem mass spectrometry, General unknown screening, Linear ion trap

1. Introduction

Recent reviews have underlined the emerging role of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in therapeutic drug monitoring and in clinical and forensic toxicology (1–6). This technology is increasingly used as a complement to immunoassays, high performance liquid chromatography (HPLC)-diode array detection (DAD) and gas chromatography (GC)-mass spectrometry (MS) for screening purposes. Two principal strategies are generally applied in this aim with LC-MS/MS.

The first one is based on multiple reaction monitoring (MRM) methods, allowing the search of quite a high number of compounds within a single run (7). However, this technology (generally called "multi-target screening") is restricted to the identification of compounds included in a predefined list, and is hardly compatible with the general recommendation of monitoring at least two specific transitions per compound. Indeed, when following a single transition per compound, particularly in drug classes with many compounds of close chemical structures (e.g., tricyclic antidepressants and phenothiazines),

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interferences can flaw the identification (or the quantitation) process (8, 9). Additionally, other guidelines have sometimes been neglected by the authors of such techniques, such as the investigation of potential interferences and of ion suppression or enhancement.

The second strategy, allowing untargeted and more specific compound identification, has emerged with the introduction of the QTRAP™ technology (a tandem mass spectrometer whose third quadrupole can be used as a linear ion trap (10)). This strategy uses information-dependent acquisition (IDA), an artificial intelligence program, which detects the most abundant ions in a "survey" scan mode and automatically and immediately switches to a "dependent" scan mode, in which the ions are transmitted to the collision cell and the resulting fragments analyzed in the third quadrupole. This approach, called a "general unknown screening procedure" can be used for the search and specific identification of unexpected compounds (11). In these conditions, compounds are ideally identified by comparison of the EPI-MS/MS spectra obtained with those generated by injecting pure compounds in identical MS conditions and entered in libraries. However, certain compounds (e.g., metabolites) with no match in the libraries can also be tentatively identified based on their mass and spectrum similarity with similar chemicals (e.g., their parent compound) and further confirmed if necessary using different MS techniques or by comparison with pure compounds. For instance, metabolites often show common mass fragments with their parent compound and an easily identifiable switch in molecular mass. Their detection in blood and above all urine can be of the utmost importance for the confirmation of drug intake (12, 13).

Alternatively, Weinmann et al. have used MRM as the survey mode (14), targeting 700 compounds owing to the introduction of the scheduled MRM application (15). However, this latter hybrid technique ("multi-target screening" for the first step and "full scan identification" for the second) cannot be regarded as a general unknown screening, as it can only detect compounds included in a predefined list.

We describe here our LC-MS/MS general unknown screening procedure, based on a nonspecific solid-phase extraction, reverse-phase HPLC, full-scan detection and full-scan identification, which has been proved to elicit the identification of drugs from many different therapeutic classes and some of their metabolites (11).

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ at 25°C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1. Reagents

1. Internal standard stock solution: 1 g/L Glafenine. Weigh 10 mg of glafenine (Sigma, or equivalent) in a brown recipient and add 10 mL of water. Mix until a perfect dissolution is observed (see Note 1). Store at –20°C.
2. 10 mg/L Glafenine solution. Prepare daily for use by diluting 50 µL of the stock solution in 4.95 mL of water.
3. Methanol: water (90:10, by volume). Mix 450 mL of methanol and 50 mL of water.
4. Dichloromethane:isopropanol:formic acid. Mix 375 mL of dichloromethane and 125 mL of isopropanol. Transfer 98 mL of this solution to a bottle and add 2 mL formic acid (>98% pure).
5. Saturated zinc sulfate solution (50 g/L). Add about 100 mL water to a 1 L glass beaker. Weigh 50 g of zinc sulfate and transfer to the beaker. Mix and make up to 1 L with water.
6. Saturated zinc sulfate:methanol (70:30, by volume). Mix 350 mL of the saturated zinc sulfate solution and 150 mL of methanol.
7. 0.5 mM ammonium formate buffer (pH 3.0). Add about 100 mL of water to a 1 L glass beaker. Weigh 31.5 mg of ammonium formate and transfer to the glass beaker. Make up to 1 L with water, mix, and adjust pH with formic acid (>98% pure).
8. 2 mM ammonium formate buffer (pH 3.0). Add about 100 mL of water to a 1 L glass beaker. Weigh 126 mg of ammonium formate and transfer to a glass beaker. Make up to 1 L with water, mix, and adjust pH with formic acid (>98% pure).
9. 10 mM ammonium formate buffer (pH 3.0). Add about 100 mL of water to a 1 L glass beaker. Weigh 630 mg of ammonium formate and transfer to a glass beaker. Make up to 1 L with water, mix, and adjust pH with formic acid (>98% pure).
10. Acetonitrile: 10 mM ammonium formate buffer (90:10, by volume). Mix 900 mL of acetonitrile and 100 mL of the 10 mM ammonium formate buffer.
11. Acetonitrile: 2 mM ammonium formate buffer (30:70, by volume). Mix 30 mL of acetonitrile and 70 mL of the 2 mM ammonium formate buffer.

2.2. Equipment

1. Oasis HLB extraction cartridges.
2. XTerra MS C18 3.5 µm (100×2.1 mm) analytical column.
3. High-pressure gradient pumping system and a Rheodyne Model 7725 injection valve equipped with a 20-µL internal loop.
4. Hybrid quadrupole/linear ion trap mass spectrometer QTRAP™, or equivalent mass spectrometer.

3. Methods

3.1. Sample Extraction: Serum, Plasma, and Urine Samples

1. Install HLB cartridges on the vacuum manifold.
2. Add 2 mL of methanol, wait at least 1 min, and then open the tap. Let the drops flow with gravity.
3. Add 2 mL of water. Let the drops flow with gravity. Close the tap after the end of water elution.
4. Fill a 1.5 mL Eppendorf tube with 1 mL of sample. Add 100 µL of the 10 mg/L glafenin solution. Mix and centrifuge at 16,000×*g* for 5 min.
5. Apply the supernatant to the cartridge. Wait at least 1 min, and then open the tap. Let the drops flow with gravity.
6. Add 3 mL of water. Let the drops flow with gravity.
7. Add 3 mL of 90:10 methanol:water. Let the drops flow with gravity.
8. Dry under vacuum for at least 15 min (see Note 2).
9. Close the tap, and put a glass tube under each cartridge. Add 3 mL of the dichloromethane:isopropanol:formic acid mixture, wait at least 1 min, and then open the tap. Let the drops flow with gravity (see Note 3).
10. Dry under vacuum for 1 min.
11. Evaporate the eluate at 40°C with a low flow-rate of nitrogen (see Note 4).
12. Add 100 µL of the 30:70 acetonitrile: 2 mM ammonium formate buffer mixture. Vortex for 30 s.
13. Transfer the solution to an injection vial.

3.2. Sample Extraction: Whole Blood, Gastric Contents, Spleen, Liver, or Brain Homogenates

1. Install HLB cartridges on the vacuum manifold.
2. Add 2 mL of methanol and then open the tap. Let the drops flow with gravity.
3. Add 2 mL of water and let the drops flow with gravity.
4. Fill a glass tube with 1 mL of sample and 2 mL of the 70:30 saturated zinc sulfate:methanol mixture. Add 100 µL of the 10 mg/L glafenin solution. Mix and centrifuge at 16,000×*g* for 5 min (see Note 5).
5. Transfer the supernatant to another glass tube and add 4 mL of water. Mix and centrifuge at 16,000×*g* for 5 min (see Note 6).
6. Apply the supernatant to the cartridge. Wait at least 1 min, and then open the tap. Let the drops flow with gravity.
7. Add 3 mL of water. Let the drops flow with gravity.

8. Add 3 mL of 90:10 methanol:water. Let the drops flow with gravity.
9. Dry under vacuum for at least 15 min (see Note 2).
10. Close the tap, and put a glass tube under each cartridge. Add 3 mL of the dichloromethane:isopropanol:formic acid mixture, wait at least 1 min, and then open the tap. Let the drops flow with gravity (see Note 3).
11. Dry under vacuum for 1 min.
12. Evaporate the eluate at 40°C with a low flow-rate of nitrogen (see Note 4).
13. Add 100 µL of the 30:70 acetonitrile: 2 mM ammonium formate buffer mixture. Vortex for 30 s.
14. Transfer the solution to an injection vial (see Note 7).

3.3. Liquid Chromatography

1. Install a Waters XTerra MS C18, 3.5 µm (100×2.1 mm) on the analytical system. A pre-column can be used to protect the analytical column if desired.
2. Mobile phase A (MP-A): 0.5 mM ammonium formate buffer (pH 3.0).
3. Mobile phase B (MP-B): 90:10 acetonitrile: 10 mM ammonium formate buffer.
4. Apply the following gradient at ca. 25°C and a constant flow-rate of 200 µL/min:
   (a) 0–2.5 min: 3% MP-B.
   (b) 2.5–21.5 min: linear increase to 90% MP-B.
   (c) 21.5–23 min: hold at 90% MP-B.
   (d) 23–23.5 min: linear decrease to 3% MP-B.
   (e) 23.5–30 min: equilibrate at 3% MP-B.
5. Inject 20 µL (see Note 8).

3.4. Mass Spectrometry

1. Use the following parameters for the enhanced mass scan (EMS) mode (see Note 9):
   (a) Ion spray voltage (IS): 5,000 V.
   (b) Declustering potential (DP): 50 V.
   (c) Curtain gas (CUR): 20 units.
   (d) Ion source gas 1 (GS1): 15 units.
   (e) Ion source gas 2 (GS2): 30 units.
   (f) Collision energy (CE): 5 V.
   (g) Collision-activated dissociation (CAD) gas: High.
   (h) Scan from 100 to 1,100 amu at a scan rate of 4,000 amu/s.
   (i) Dynamic fill time: On.

2. Use the following parameters for the enhanced product ion (EPI) scan mode (see Note 10):
   (a) Ion spray voltage (IS): 5,000 V.
   (b) Declustering potential: 50 V.
   (c) CUR: 20 units.
   (d) GS1: 15 units.
   (e) GS2: 30 units.
   (f) Entrance potential (EP): 10 V.
   (g) Collision energy: 40 V.
   (h) Collision energy spread (CES): 25 V.
   (i) CAD gas: High.
   (j) Scan from 50 to 1,100 amu at a scan rate of 4,000 amu/s.
   (k) Dynamic fill time: On (see Note 11).
3. IDA conditions:
   (a) Select the three most intense ions in the EMS mode.
   (b) Apply dynamic background subtraction (see Note 12).
   (c) Do not set any intensity threshold.
   (d) Exclude for 15 s all ions previously selected for four consecutive occurrences (see Note 13).

3.5. Library Search

1. Using the analyst software and the "IDA Explorer to display IDA samples" feature, select the acquired files using "Open data file" (see Note 14 and Fig. 1).
2. Export the EPI spectrum to the Explorer using the predefined icon.
3. Check the search constraints by right-clicking (Window F in Fig. 1).
4. Perform library search by right-clicking in the EPI spectrum window.
5. Evaluate the purity value obtained (Window J in Fig. 1): a purity of at least 90% is required for the positive identification of a compound (see Note 15).

3.6. Graphical Representation of the Acquisition Procedure

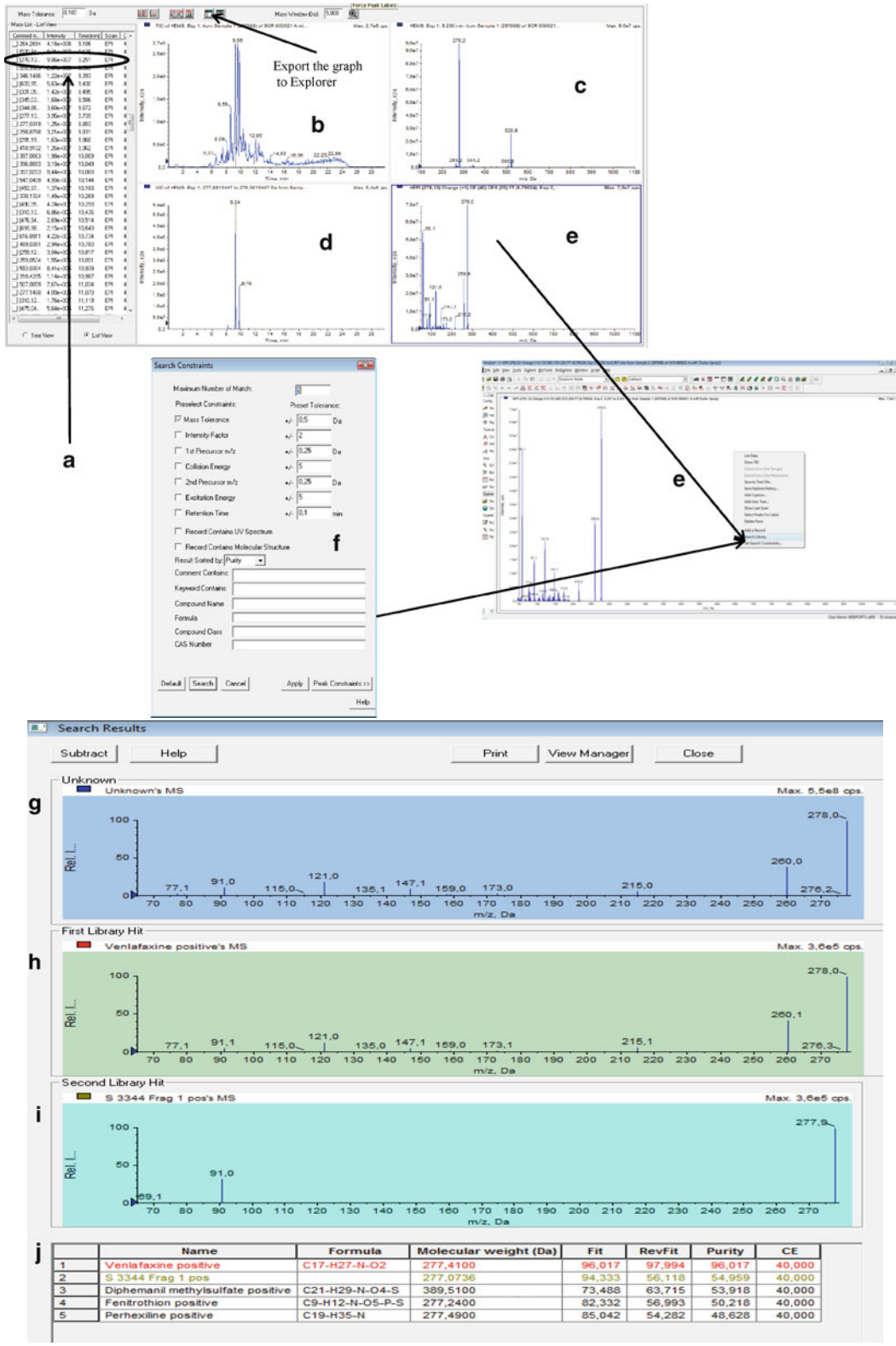
The different steps of the acquisition procedure are graphically represented in four windows (Fig. 2):

Window A: Total ion current of the survey scan mode.

Window B: Extracted ion chromatogram obtained after the selection of the ion with m/z 264.0.

Window C: m/z values of the ions observed at 8.1 min.

Window D: EPI spectrum of the selected ion.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Venlafaxine positive	C17-H27-N-O2	277,4100	96,017	97,994	96,017	40,000
2	S 3344 Frag 1 pos		277,0736	94,333	56,118	54,959	40,000
3	Diphemanil methylsulfate positive	C21-H29-N-O4-S	389,5100	73,488	63,715	53,918	40,000
4	Fenitrothion positive	C9-H12-N-O5-P-S	277,2400	82,332	56,993	50,218	40,000
5	Perhexiline positive	C19-H35-N	277,4900	85,042	54,282	48,628	40,000

Fig. 1. (**a**) Mass list, (**b**) Total ion current of the survey scan mode, (**c**) Underlying Q3-only enhanced mass spectrum (EMS) at 9.2 min, (**d**) Extracted ion chromatogram of the compound with / 278.0, (**e**) Enhanced product ion spectrum of the selected compound in a forensic blood sample, (**f**) list of search constraints, (**g**) unknown spectrum window, (**h**) first library hit window, (**i**) second library hit window, (**j**) library search results.

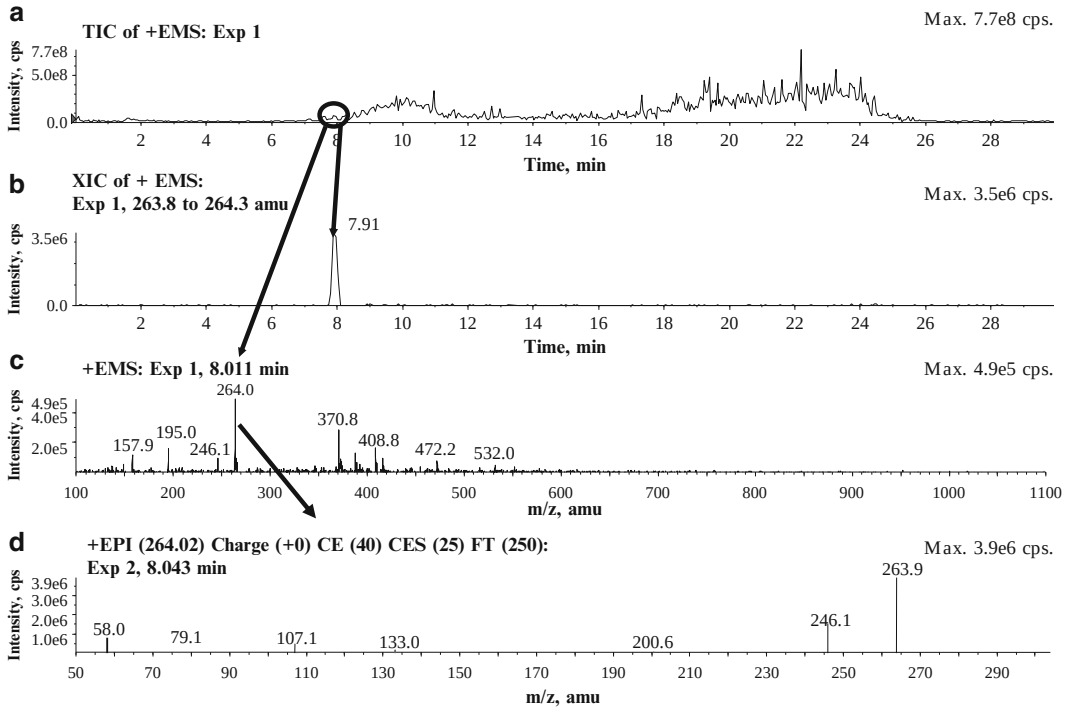


Fig. 2. (**a**) Total ion chromatogram, (**b**) Extracted ion chromatogram of the compound with *m/z* 264.0, (**c**) Underlying Q3-only enhanced mass spectrum (EMS) at 8.0 min, (**d**) Enhanced product ion spectrum of the selected compound in a forensic blood sample.

4. Notes

1. Small particles of glafenine can appear at the bottom of the bottle after freezing. A better strategy is to aliquot the stock solution in several microtubes.
2. A longer drying time allows evaporation of the remaining water after elution.
3. The color of the solid-phase changes and little bubbles can be observed in the discarded solvent.
4. Do not use higher temperatures, as some compounds like benzodiazepines can be destroyed.
5. Due to the viscosity of this kind of samples, protein precipitation can hardly be avoided.
6. Because methanol was employed for protein precipitation, addition of water to the supernatant is necessary to elicit compound retention on the cartridge.
7. The sample can be very dirty after evaporation and may require an extra step of centrifugation before injection. It avoids problems of increasing backpressure.

8. 20 µL can be injected on a 2000QTRAP™ instrument. Smaller volumes may be sufficient with 3200QTRAP™ or 4000QTRAP™ instruments.
9. The collision energy is fixed at its lowest value in order to avoid fragmentation of the parent ions.
10. The use of the collision energy spread generally results in EPI spectra presenting the pseudo-molecular ion of the investigated compound, due to the use of low CE, as well as some other fragments due to higher values of CE.
11. The EPI spectrum can be saturated for highly concentrated compounds. Hence, it is preferable to use the dynamic LIT fill time option, which prevents saturation of the linear ion trap.
12. On the last released versions of the Analyst software, dynamic background subtraction can be selected with a single click. An independent script is needed for older versions.
13. The automatic exclusion of formerly observed compounds helps detect co-eluting compounds.
14. The windows observed after opening a general unknown screening data are presented in Fig. 1.
    (a) Window A: List of the centroid *m/z* and their respective intensity, retention time, scan type, and collision energy (this display can be modified using the "IDA Explorer Appearance Options").
    (b) Window B: Total ion current of the survey scan mode.
    (c) Window C: *m/z* values of the ions observed at the selected retention time.
    (d) Window D: Extracted ion chromatogram of the selected *m/z* ratio.
    (e) Window E: EPI spectrum of the selected parent *m/z*.
    (f) Window F: List of the search constraints which can be selected for library search.
    (g) Window G: EPI spectrum selected for the library search considered as unknown spectrum.
    (h) Window H: library EPI spectrum with the best fit (first library hit).
    (i) Window I: EPI spectrum corresponding to the second library hit.
    (j) Window J: List of the compounds with the five best hits (which can be ranked by fit, reverse fit or purity—ranked here by decreasing purity).
15. Search results are sorted by three criteria: (a) purity, measured as the unmatched peaks between the unknown and known spectra; (b) fit, measured by how well a library spectrum

matches the unknown spectrum; and (c) reverse fit, which measures how well the unknown spectrum matches a library spectrum. All these criteria range from 0 to 100%.

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Chapter 12

Qualitative Identification of Rodenticide Anticoagulants by LC-MS/MS

Robert A. Middleberg and Joseph Homan

Abstract

Rodenticide anticoagulants are used in the control of rodent populations. In addition to accidental ingestions in humans, such agents have also been used for homicidal and suicidal purposes. There are two major groups of rodenticide anticoagulants – hydroxycoumarins and indanediones. Before the advent of LC-MS/MS, analysis for such agents was relegated to such techniques as TLC and HPLC with nonspecific modes of detection. LC-MS/MS has been used to determine any given number of rodenticide anticoagulants in animal tissues, foods, plasma, etc. Use of this technique allows for the simultaneous identification of individual compounds within both classes of rodenticide anticoagulants. The LC-MS/MS method presented allows for simultaneous qualitative identification of brodifacoum, bromadiolone, chlorphacinone, dicumarol, difenacoum, diphacinone, and warfarin in blood, serum, and plasma using ESI in the negative mode. Two transitions are monitored for each analyte after a simple sample preparation. Chromatographic separation is accomplished using a gradient of ammonium hydroxide in water and ammonium hydroxide in methanol. Chloro-warfarin is used as internal standard.

Key words: Rodenticide anticoagulants, Blood, Serum, Plasma, LC-MS/MS, Negative ESI, Qualitative

1. Introduction

Rodenticide anticoagulants are used today to help control pest populations. Many such preparations can be easily accessed in various stores where pest control products are sold. Often, such products are packaged as pellet or candy-shaped forms in bright colors, thus facilitating accidental ingestion in humans, especially children. As resistance has developed in rodents to first-generation rodenticide anticoagulants, e.g., warfarin, newer, more potent second-generation "superwarfarins" have been developed. However, this latter development has also led to increased risk to humans as single doses can elicit toxicity (1). Additionally, due to their ready availability and potency, rodenticide anticoagulants have been used

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in both homicidal and suicidal attempts in humans and domesticated pets (2, 3). The newer superwarfarins, e.g., brodifacoum and difenacoum, are particularly insidious due to their rather long half-lives, therefore making exposure treatment difficult to control. Biochemically, these compounds have two significant actions: inhibition of synthesis of vitamin K-dependent clotting factors and inhibition of prothrombin in the liver (4). Signs and symptoms of such exposure usually develop within a day or so of exposure and include nose and gum bleeding, hematuria, melena, ecchymoses, and if left untreated, potentially shock and death (1).

Attempts at identifying rodenticide anticoagulants in biological fluids and tissues were quite challenging initially. Early analytical techniques following extraction included thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (5–9). Mass spectral identification of rodenticide anticoagulants was performed relatively early in the maturation of mass spectrometry in the clinical world (6). The advent of liquid-chromatography tandem mass spectrometry (LC-MS/MS) has led to significant improvements in the analysis of rodenticide anticoagulants in a diverse range of biological specimens (10–12).

Blood, serum, and plasma present unique challenges in respect to analyte isolation and sensitivity requirements in both a general sense and specific to the rodenticide anticoagulants, thus making LC-MS/MS a logical choice for instrumental analysis.

A simple extraction procedure was developed to isolate the following rodenticide anticoagulants: brodifacoum; bromadiolone; chlorphacinone; dicumarol; difenacoum; diphacinone; and warfarin. An analog internal standard, chloro-warfarin, was used in the assay. Chromatographic separation of the rodenticide anticoagulants was facilitated using ultra performance liquid chromatography (UPLC) employing a reverse phase column and a mobile phase gradient. Mass spectrometric analysis employed electrospray ionization in the negative mode with two transitions accumulated for each analyte.

2. Materials

2.1. Reagents (All HPLC or ACS Grade)

(a) Mobile phase A (MP-A): 0.02% ammonium hydroxide in DI water.

(b) Mobile phase B (MP-B): 0.02% ammonium hydroxide in methanol.

(c) Water saturated methyl-*t*-butyl ether (MTBE).

(d) Reconstitution solution: 80% mobile phase A, 20% mobile phase B.

2.2. Standard Reference Materials

(a) Warfarin (Sigma, St. Louis, MO).
(b) Dicumarol (Sigma, St. Louis, MO).
(c) Diphacinone (Chem Service, West Chester, PA).
(d) Chlorphacinone (Chem Service, West Chester, PA).
(e) Difenacoum (Pfaltz & Bauer, Inc., Waterbury, CT).
(f) Bromadiolone (Chem Service, West Chester, PA).
(g) Chloro-Warfarin (Sigma, St. Louis, MO).

2.3. Stock Standard

Prepare 1,000 ng/μL stock standards of brodifacoum, bromadiolone, chlorphacinone, dicumarol, difenacoum, diphacinone, and warfarin by dissolving the appropriate weight of material in acetone. Store the stock standards at −10°C in amber glass bottles with Teflon-lined caps. Stock standards can be verified using UV spectrophotometry and Beer's Law (see Note 1).

2.4. Rodenticide Anticoagulants Mixed Standard

Pipette 100 μL of each stock standard solution into a 10 mL volumetric flask. Dilute to volume with acetonitrile for a final concentration of 10 ng/μL of each analyte. Store this solution in a Teflon-lined capped amber vial at −10°C.

2.5. Rodenticide Anticoagulants Mixed Sub-stock Standard

Pipette 10 μL of each stock standard solution into a 10 mL volumetric flask. Dilute to volume with acetonitrile for a final concentration of 1 ng/μL of each analyte. Store this solution in a Teflon-lined capped amber vial at −10°C.

2.6. Rodenticide Anticoagulants Working Standard

Transfer a 0.20 mL aliquot of blank serum or blood to an appropriately labeled 12×75 mm tube. Add 2 μL of the rodenticide anticoagulants mixed sub-stock standard. This 10 ng/mL "calibrator" serves as the reporting limit check for the assay.

2.7. Internal Standard

(a) Prepare an individual 100 ng/μL stock solution of chlorowarfarin.
(b) From the stock solution in Subheading 2.7, item (a), prepare an internal standard working solution by transferring 250 μL of the 100 ng/μL stock solution into a 25 mL volumetric flask and bringing to volume with methanol. Store this solution in a Teflon-lined capped amber vial at −10°C.

2.8. Control

(a) Prepare individual stock quality control (QC) solutions as described in Subheading 2.3 above. Store these solutions in Teflon-lined capped amber vials at −10°C.
(b) Transfer 100 μL of each individual stock QC solution (from Subheading 2.8, item (a)) to separate 10 mL volumetric flasks and bring to volume with acetonitrile. Vortex to mix.
(c) Transfer 100 μL of each solution in Subheading 2.8, item (b) to a 50 mL volumetric flask containing enough room temperature

serum or blood to cover the bottom of the volumetric flask. Dilute to volume with the appropriate matrix. This is a 20 ng/mL mixed control.

2.9. Equipment

(a) The instrument employed is a Waters Micromass Quattro Premier TQD Mass Spectrometer with a Waters Acquity Ultra Performance LC equipped with Mass Lynx software.

(b) Column: waters acquity UPLC BEH C18 1.0 mm ID × 50 mm, 1.7 μm.

3. Methods

Due to the use of chemicals and material of biological origin, procedures consistent with a laboratory's chemical hygiene and bloodborne pathogens standard operating procedures must be followed.

3.1. Sample Preparation

1. Transfer 200 μL blank matrix (serum or blood), working standard, controls, and patient specimens to appropriately labeled 12 × 75 mm tubes (see Notes 2 and 3).
2. Add 25 μL of working internal standard to each tube; vortex to mix.
3. Add 300 μL of acetonitrile to each tube; vortex to mix.
4. Centrifuge for 5 min at approximately 1,740 × *g*. Decant the supernatant to a set of appropriately labeled 13 × 100 mm tubes.
5. Add 2.0 mL of water-saturated MTBE to each tube; vortex well to mix.
6. Centrifuge for approximately 5 min at approximately 1,740 × *g*.
7. Transfer upper layer to appropriately labeled 12 × 75 mm tubes. This can be facilitated by freezing the tubes in a dry ice/acetone bath and decanting the top organic layer or by pipetting the top layer.
8. Evaporate to dryness at 40°C ± 5°C under nitrogen.
9. Reconstitute with 150 μL of 80% mobile phase A/20% mobile phase B.
10. Vortex well and transfer to appropriately labeled autosampler vials. Seal with Teflon®-lined pre-slit caps or equivalent. Extracts are ready for LC-MS/MS analysis. Validation studies have shown samples are stable in autosampler vials for no longer than 24 h; samples must be injected within 24 h of preparation (see Note 4).

3.2. Instrument Parameters

1. Instrument parameters—see Table 1.
2. Method parameters—see Table 2.
3. Autosampler parameters—see Table 3.

Table 1
Instrument parameters

Parameter	Settings
Polarity	ES-
Capillary (kV)	0.80
Cone (V)	28.00
Extractor (V)	2.00
RF (V)	0.00
Source temperature (°C)	120
Desolvation temperature (°C)	450
Cone gas flow (L/h)	35
Desolvation gas flow (L/h)	700
Collison gas flow (mL/min)	0.20
LM 1 resolution	13.00
HM 1 resolution	13.00
Ion energy 1	0.50
MS mode entrance	50.00
MS mode collision energy	1.00
MS mode exit	50.00
MSMS mode entrance	−5.00
MSMS mode collision energy	25.00
MSMS mode exit	1.00
LM 2 resolution	13.00
HM 2 resolution	13.00
Ion energy 2	2.00
Gain	1.00
Multiplier	−0.52
Active reservoir	A
Autotune reservoir	B
Collision cell pressure (mbar)	5.67

Table 2
Method parameters

Parameter	Value
Waters acquity SDS	
Run time	2.50 min
Comment	
Solvent selection A	A2
Solvent selection B	B2
Low pressure limit	0 psi
High pressure limit	15,000 psi
Solvent name A	0.02% Ammonium hydroxide in water
Solvent name B	0.02% Ammonium hydroxide in methanol
Switch 1	No change
Switch 2	No change
Switch 3	No change
Seal wash	5.0 min
Chart out 1	System pressure
Chart out 2	% B
System pressure data channel	No
Flow rate data channel	No
% A data channel	No
% B data channel	No
Primary A pressure data channel	No
Accumulator A pressure data channel	No
Primary B pressure data channel	No
Accumulator B pressure data channel	No
Degasser pressure data channel	No

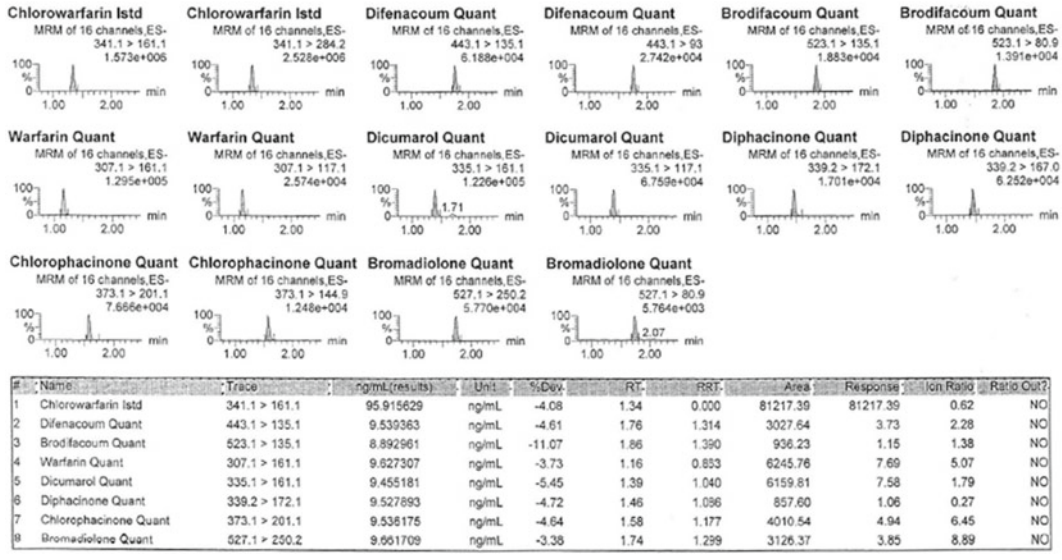
4. Flow rate = 0.2 mL/min. Mobile phase gradient:
   Initial: 5% MP-B.
   0–1.6 min: linear gradient to 95% MP-B.
   1.6–2 min: hold at 95% MP-B.
   2–2.1 min: reduce to 5% MP-B.
   2.1–2.5 min: hold at 5% MP-B.
5. Sample MRM data—see Fig. 1.

3.3. Method of Calculation

1. The ions monitored for internal standard and analytes are listed in Table 4.
2. Each analyte must meet relative retention time parameters (±2% of calibrators) and ratio characteristics (±30% of calibrators). Relative retention time is defined as the retention time of the analyte divided by the retention time of the internal standard. The ratio characteristic is defined as the ratio of the

Table 3
Waters acquity autosampler parameters

Parameter	Value
Run time	2.50 min
Load ahead	Disabled
Loop option	Partial loop
Loop offline	Disable
Weak wash solvent name	Water
Weak wash volume	600 µL
Strong wash solvent name	Methanol
Strong wash volume	500 µL
Target column temperature	60.0°C
Column temperature alarm band	5.0°C
Target sample temperature	Off
Sample temperature alarm band	Disabled
Full loop overfill factor	Automatic
Syringe draw rate	Automatic
Needle placement	1.0
Pre-aspirate air gap	Automatic
Post-aspirate air gap	Automatic
Column temperature data channel	No
Ambient temperature data channel	No
Sample temperature data channel	No
Sample organizer temperature data channel	No
Sample pressure data channel	No
Switch 1	No change
Switch 2	No change
Switch 3	No change
Switch 4	No change
Chart out	Sample pressure
Sample temp alarm	Disabled
Column temp alarm	Enabled
Run events	Yes
Needle overfill flush	Automatic
Injection volume	20.0 µL



#	Name	Trace	ng/mL(results)	Unit	%Dev.	RT	RRT	Area	Response	Ion Ratio	Ratio Out?
1	Chlorowarfarin Istd	341.1 > 161.1	95.915629	ng/mL	-4.08	1.34	0.000	81217.39	81217.39	0.62	NO
2	Difenacoum Quant	443.1 > 135.1	9.539363	ng/mL	-4.61	1.76	1.314	3027.64	3.73	2.28	NO
3	Brodifacoum Quant	523.1 > 135.1	8.892961	ng/mL	-11.07	1.86	1.390	936.23	1.15	1.38	NO
4	Warfarin Quant	307.1 > 161.1	9.627307	ng/mL	-3.73	1.16	0.853	6245.76	7.69	5.07	NO
5	Dicumarol Quant	335.1 > 161.1	9.455181	ng/mL	-5.45	1.39	1.040	6159.81	7.58	1.79	NO
6	Diphacinone Quant	339.2 > 172.1	9.527893	ng/mL	-4.72	1.46	1.096	857.60	1.06	0.27	NO
7	Chlorophacinone Quant	373.1 > 201.1	9.536175	ng/mL	-4.64	1.58	1.177	4010.54	4.94	6.45	NO
8	Bromadiolone Quant	527.1 > 250.2	9.661709	ng/mL	-3.38	1.74	1.299	3126.37	3.85	8.89	NO

Fig. 1. Sample MRM data.

Table 4
Quantifier and qualifier transitions

Analyte	Quant ion	Ratio ion
Chloro-Warfarin	341.1 > 161.1	341.1 > 284.2
Brodifacoum	523.1 > 135.1	523.1 > 80.9
Bromadiolone	527.1 > 250.2	527.1 > 80.9
Chlorphacinone	373.1 > 201.1	373.1 > 144.9
Dicumarol	335.1 > 161.1	335.1 > 117.1
Difenacoum	443.1 > 135.1	443.1 > 93
Diphacinone	339.2 > 172.1	339.2 > 167.0
Warfarin	307.1 > 161.1	307.1 > 117.1

response of one transition of the analyte of interest to that of another transition for that analyte. This ratio should be relatively constant from sample to sample. The matrix blank must have response ratios less than that seen in the 10 ng/mL calibrator for all analytes.

4. Notes

1. The use of Beer's Law can be used to determine the concentration of a solution via use of the molar absorptivity constant (ε). Classically, the following formula is applied:

   $C = A/\varepsilon b$,

   where C = concentration,

   A = absorbance,

   ε = molar absorptivity coefficient,

   b = path length.

   Molar absorptivity constants for various substances in a given solution at a given concentration can be found in many references, e.g., Merck Index.
2. Working standards are run in the beginning and end of a batch. The response of these calibrators is averaged for purposes of determining where patient samples are determined to be positive for a given rodenticide anticoagulant.
3. All patient samples are run as standard additions. That is, a separate aliquot of a patient sample is spiked with the analytes of interest so that each is present at a concentration of 20 ng/mL. This process controls for recovery and matrix-related issues.
4. The nature of the autosampler stability issue is undetermined, especially in a qualitative analysis. It is possible that the analytes adsorb to plastic or crystallize under these reconstitution conditions.

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Chapter 13

Hypoglycemic Agent Screening by LC-MS/MS

Eric W. Korman, Loralie J. Langman, and Christine L.H. Snozek

Abstract

Hypoglycemia is a potentially life-threatening condition that can be caused by a variety of physiological conditions and pharmaceutical agents. Diagnosis of certain conditions, such as insulin-secreting tumors, requires ruling out the use (inadvertent or surreptitious) of drugs capable of inducing hypoglycemia. Many of the therapeutic agents used to treat diabetes mellitus have the ability to lower blood glucose to dangerous concentrations; these include the sulfonylurea, meglitinide, and thiazolidinedione drug classes. The LC-MS/MS assay presented herein provides qualitative or quantitative assessment of the most common of these drugs, for assistance in the differential diagnosis of hypoglycemia.

Key words: Hypoglycemia, Insulinoma, Sulfonylureas, Meglitinides, Thiazolidinediones

1. Introduction

Hypoglycemic agents comprise a sizeable group of drugs used therapeutically to prevent diabetes-related hyperglycemia. Insulin is an endogenous hypoglycemic agent: when present in excess relative to the blood glucose concentration, insulin induces tissue uptake of glucose from the blood. In healthy individuals, this process is tightly regulated, but disease states such as diabetes mellitus, insulin-releasing tumors (i.e., insulinomas), and a variety of endocrine disorders (e.g., adrenal insufficiency), can drastically disrupt normal blood glucose balance (1).

Exogenous insulin can therapeutically lower elevated glucose levels in insulin-responsive diabetics. However, administration of excessive insulin bears the risk of inducing hypoglycemia, a state characterized by malaise, trembling or convulsions, irritability, confusion, and possible coma or death (2). Similarly, there are a wide array of drugs that mimic or complement the glucose-lowering properties of insulin; many of these therapeutic agents are also associated with the risk of hypoglycemia.

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Iatrogenic hypoglycemia from sources other than exogenous insulin is most commonly seen with drugs of the sulfonylurea or meglitinide classes, and to a lesser degree with thiazolidinediones (3–5). Some drugs used to treat diabetes are not associated with hypoglycemia unless combined with another hypoglycemic agent; examples include metformin and exenatide (2). Older (first-generation) sulfonylurea drugs are rarely prescribed in modern practice, as they appear to have the greatest risk of inducing hypoglycemia; these include acetohexamide and chlorpropamide. Later (second- or third-generation) sulfonylureas such as glipizide and glimepiride can cause hypoglycemia, but are less likely to do so compared to the older agents (2).

The method described here detects and quantitates sulfonylurea, meglitinide, and thiazolidinedione drugs available in the United States. Analysis of these agents can be performed to assess compliance and therapeutic response. However, a more critical use of this test is to assist in the differential diagnosis of unexpected hypoglycemia. Surreptitious or inadvertent use of hypoglycemic agents can mimic the presence of an insulinoma; such tumors can be difficult to find with imaging techniques, thus screening for hypoglycemic agent use is prudent prior to performing exploratory surgery or other serious medical interventions (6, 7).

2. Materials

2.1. Solvents and Chemicals

1. All reagents are of HPLC or analytical grade.
2. Analyte stocks (as powder): Nateglinide, pioglitazone HCl, rosiglitazone maleate (all from Toronto Research Chemical); acetohexamide, chlorpropamide, glimepiride, glipizide, glyburide, tolazamide, tolbutamide, repaglinide (all from Sigma).
3. Internal standards (as powder): Glimepiride-d_5, repaglinide ethyl-d_5, rosiglitazone-d_3 (all from Toronto Research Chemical); zomepirac (Sigma).

2.2. Prepared Reagents

1. Extraction solvent: mix equal volumes of ethyl acetate and hexane.
2. Mobile phase A (MP-A): 20 mM ammonium acetate in 95:5 water:methanol. Add 50 mL of methanol to 900 mL of Type I water in a 1 L volumetric flask. Add 1.54 g of ammonium acetate and mix to dissolution. Bring to volume with Type I water.
3. Mobile phase B (MP-B): 100% methanol.
4. Reconstitution solution: 90:10 water:methanol. Prepare with Type I water.
5. 0.1 M Sodium acetate buffer, pH 4.5. To a 1 L volumetric flask, add approximately 750 mL of Type I water. While stirring,

slowly add 8.2 g of sodium acetate. Once this dissolves pH to 4.5 with approximately 2.0 mL of glacial acetic acid. Allow to cool before bringing to volume with Type I water. Mix well.

6. 5.0 mg/mL methanolic stocks for standards and controls.
   (a) Standard Stock: Quantitatively transfer 50 mg of each pure drug powder (except pioglitazone HCl and rosiglitazone maleate) to its own 10 mL volumetric flask, bring to volume with methanol, and mix well. Stable up to 2 year stored at –20°C in screw-cap amber vials with rubber/Teflon septa. For pioglitazone HCl, perform the same procedure using 55 mg of the drug in a 10 mL flask; for rosiglitazone maleate use 66 mg of the drug in a 10 mL flask.
   (b) Control Stock: Repeat item 6a using different lots of stock standards and/or prepared independently.
7. 100 µg/mL methanolic stocks for internal standards. Quantitatively transfer 1.0 mg of each internal standard powder to its own 10 mL volumetric flask, bring to volume with methanol, and mix well. Stable up to 2 year stored at –20°C in screw-cap amber vials with rubber/Teflon septa.
8. 100 µg/mL Intermediate Standard and Control.
   (a) Intermediate Standard: Quantitatively transfer 200 µL of each Standard Stock to a 10 mL volumetric flask, bring to volume with methanol, and mix well. Stable up to 2 year stored at –20°C in screw-cap amber vials with rubber/Teflon septa.
   (b) Intermediate Control: Repeat item 8a, using methanolic Control Stocks.
9. Working Internal Standard (IS). Quantitatively transfer 100 µL of zomepirac, rosiglitizone-d_3 and repaglinide ethyl-d_5; and 1,000 µL of glimepiride-d_5 internal standard stocks to a 100 mL volumetric flask, bring to volume with methanol, and mix well. Final concentrations are 1 µg/mL for glimepiride-d_5 and 0.1 µg/mL for all others. Stable up to 2 year stored at –20°C in screw-cap amber vials with rubber/Teflon septa.
10. Working Standards and Working Controls, in 50% bovine serum.
    (a) Working Standards: Add 50 mL bovine serum to five separate 100 mL volumetric flasks. To achieve working standard concentrations of 3, 10, 25, 100, and 500 ng/mL, add 3, 10, 25, 100, and 500 µL of the Intermediate Standard, respectively. Bring to volume with Type I water, mix well, and pipette into individual 1.1 mL aliquots in cryovials. Freeze at –20°C.
    (b) Working Controls: Perform procedure as described in item 10a, using 5, 50, and 250 µL of the Intermediate Control. Final working control concentrations are 5, 50, and 250 ng/mL of each compound, respectively.

2.3. Supplies and Analytical Equipment

1. Triple quadrupole or quadrupole/time-of-flight (see Note 1) mass spectrometer, e.g., ABI3200 or similar (ABI Sciex).
2. Analytical column: 50 mm × 3 mm × 5 µm Hypersil Gold C_8 (Thermo Scientific).
3. Guard column: Supelguard Discovery C_8 2 cm × 4.0 mm × 5 µm (Supelco).

3. Methods

3.1. Extraction

1. Add 1.0 mL of each standard, control, and unknown sample to appropriately labeled 16 × 150 mm borosilicate test tubes. Also aliquot 1.0 mL of blank (drug-free) serum as a carryover control.
2. Add 50 µL of working IS solution to each tube.
3. Add 200 µL of 0.1 M sodium acetate buffer to each tube (see Note 2).
4. Add 6 mL of extraction solvent to each tube.
5. Vortex for 5 min to mix.
6. Centrifuge at 2,000 × *g* for 5 min.
7. Transfer 5 mL of organic (top) phase to a clean 16 × 100 mm borosilicate tube.
8. Dry completely under air or nitrogen at 30°C.
9. Reconstitute with 125 µL reconstitution solution (see Note 3).

3.2. Analysis

1. Place the sample extracts on the autosampler in the following order (see Note 4):

   Calibration standards, in order of lowest to highest concentration.

   Negative serum (carryover) control.

   Quality controls, lowest to highest.

   Unknown samples and additional quality controls.
2. Set LC-MS/MS method to the following parameters (see Note 5):
   (a) Autosampler parameters: Can be either ambient or refrigerate temperature. Inject 40 µL per sample.
   (b) LC parameters:

   Column temperature: 50°C with a flow rate of 0.50 mL/min.

   Mobile phase program:

   0.0–1.0 min: Hold at 30% MP-B.

   1.0–7.5 min: Linear gradient to 95% MP-B.

   7.5–8.5 min: Hold at 95% MP-B.

   8.5–9.0 min: Linear gradient to 30% MP-B.

   9.0–10.0 min: Hold at 30% MP-B.

Table 1
Analytical and detection conditions for hypoglycemic agents and Internal Standards

Drug	Retention time (min.)	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Declustering potential (V)	Collision energy (V)	Internal Standard
Acetohexamide	4.07	352.2	91.1	51	55	Zomepirac
Chlorpropamide	3.82	277.1	111.1	41	45	Zomepirac
Glimepiride	6.52	491.2	352.2	54	23	Glimepiride-d_5
Glipizide	4.88	446.2	321.2	51	23	Zomepirac
Glyburide	6.27	494.2	169.1	41	47	Glimepiride-d_5
Nateglinide	6.78	318.2	69.1	66	35	Repaglinide-d_5
Pioglitazone	6.10	357.2	134.2	71	39	Rosiglitazone-d_3
Repaglinide	7.50	453.3	230.2	30	37	Repaglinide-d_5
Rosiglitazone	5.83	358.1	78.1	61	87	Rosiglitazone-d_3
Tolazamide	4.73	312.2	115.2	61	23	Zomepirac
Tolbutamide	4.52	271.1	91.1	41	47	Zomepirac
Glimepiride-d_5	6.48	496.3	357.2	46	23	n/a
Repaglinide-d_5	7.49	458.2	230.2	80	50	n/a
Rosiglitazone-d_3	5.81	361.2	138.1	60	36	n/a
Zomepirac	5.26	292.1	139.1	41	25	n/a

(c) Ion source parameters: source temp = 600°C, capillary voltage = 5,500 V, Ion Source Gas 1 and 2 = 40 psi and curtain gas = 30 psi.

(d) Mass spectrometer parameters: Compound-dependent parameters are detailed in Table 1. Figure 1 shows the chromatography of all compounds and internal standards (see Note 6).

3. For qualitative (i.e., screening) analysis, set the mass spectrometer to the following parameters:

(a) Independent Data Acquisition (IDA) criteria: Select all peaks of interest which exceed 250 cps (will detect all analytes down to an LOD of at least 3 ng/mL).

(b) Enhanced Product Ion (EPI): Scan at 4,000 Da/s for fragments from 50 Da to 500 Da and sum only a single scan.

(c) 2nd EPI scan: There must be two EPI experiments in the assay to capture overlapping peaks.

(d) Figure 2 shows a library match of an unknown hypoglycemic agent.

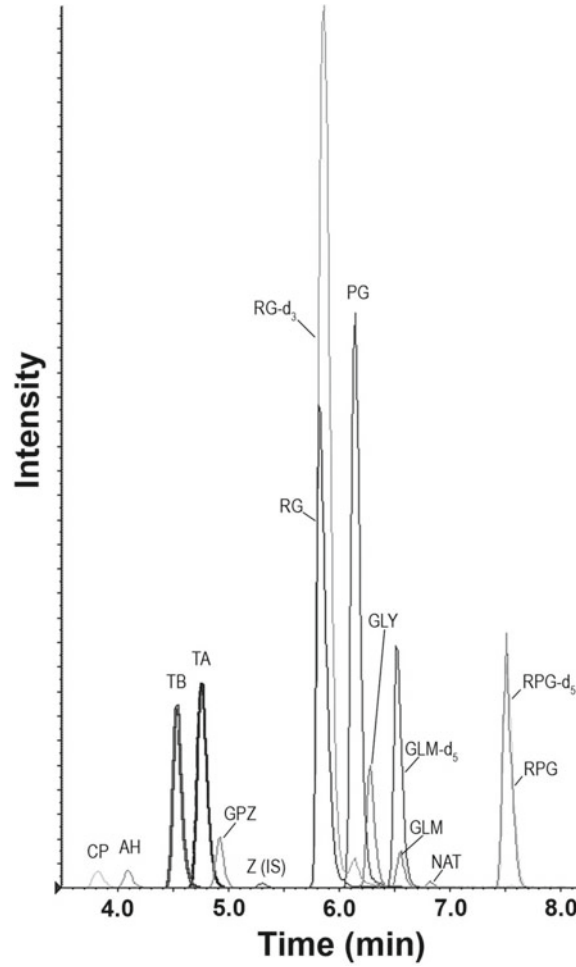

Fig. 1. Chromatography of a standard containing all compounds in the method.

4. Notes

1. This assay can be conducted with traditional multiple reaction monitoring (MRM) or also with an IDA triggered EPI which uses an ion trap in Q3 to create a total ion spectrum to compare to a library. This feature is available on instruments such as the ABI 3200 Q-Trap.
2. With so many analytes of varying chemical properties the pH of the extraction buffer is very important. Acidic pH helps to extract acetohexamide, chlorpropamide and nateglinide. However, too low of a pH hinders the extraction of rosiglitazone. The pH 4.5 buffer allows adequate extraction of all compounds for the stated limits of detection.

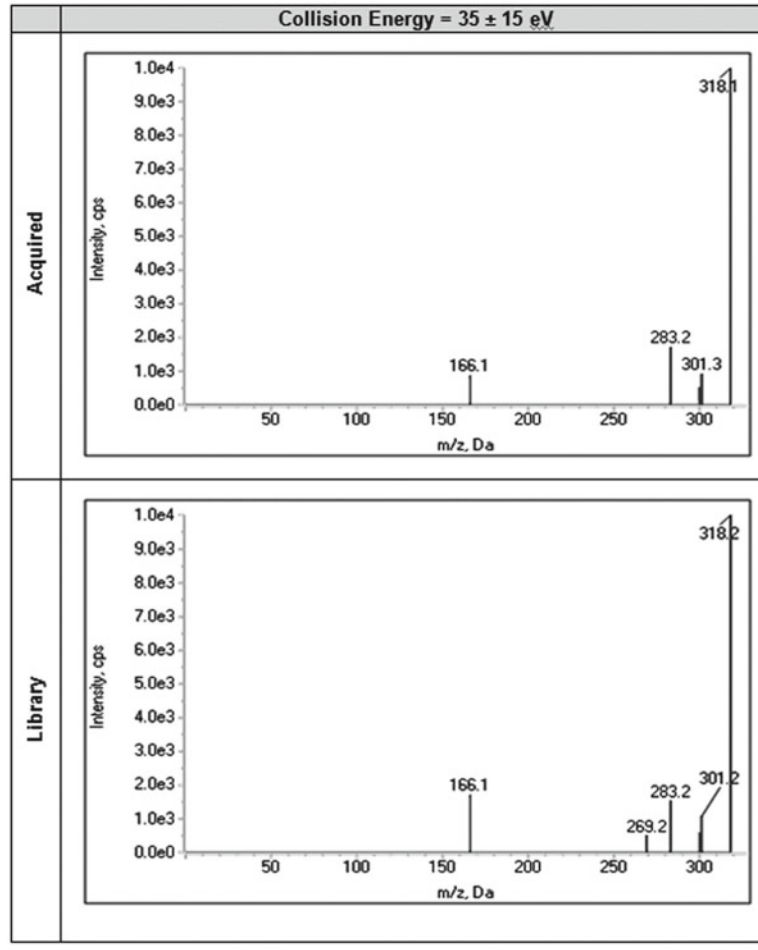

Fig. 2. EPI of unknown sample compared to library of hypoglycemic drugs.

3. Sample order is at the discretion of the user; the rationale for this order is as follows. The unextracted control confirms instrument performance independently of the success of the extraction. 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 comprises quality controls, we run one control after every nine patient samples.

4. This extraction is optimized to achieve the lowest detection limits possible, but results in a relatively dirty sample. To improve analytical column life and minimize LC-MS/MS down time, it is recommended to use a substantial C8 guard column before the analytical column, as a cost-effective means of preventing contaminants from reaching the analytical column or detector.
5. Glimepiride gives the lowest response of all of the analytes; therefore, many of the parameters such as ion spray voltage, source temperature, and mobile phase conditions were selected with the best glimepiride response in mind.
6. Not all of the analytes of this assay performed well with one internal standard; therefore, several are required for optimal performance.

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Chapter 14

LC-MS/MS Method for the Detection of Common Laxatives

Robert A. Middleberg and Joseph Homan

Abstract

Laxatives refer to a group of diverse substances used to induce bowel movements. There exist various classes of laxatives, which work through different pharmacological means. Based on the potential medical cause of use, one particular class of laxative may be preferred over another. Additionally, abuse of laxatives in both adults and children occurs. Some of the signs and symptoms of excessive laxative use/abuse can not only mimic various pathological conditions, but cause such conditions. Based on the potential abuse of laxatives, as well as for compliance purposes, a test to identify the use of common laxatives is of significant value. While stool and stool water can be used for such analyses, isolation and identification of analytes can be difficult due to matrix constituents and potential interferences. Ideally, a sensitive urine test for detection of laxative use/abuse with specific detection would be preferable. Described is an LC-MS/MS procedure for the detection of four metabolites related to common laxatives desacetylbisacodyl, aloe-emodin, emodin, and rhein. Deuterated internal standards for desacetylbisacodyl and emodin are employed while an analog internal standard, biochanin A is used for rhein and aloe-emodin. Sample preparation consists of deconjugation of analytes in urine followed by a simple organic solvent extraction. Analysis is carried out using a pentafluorophenyl column employing a gradient mobile phase of formic acid in water/methanol. Mass spectral ionization conditions employ both positive and negative ESI. Two transitions are monitored for each analyte of interest.

Key words: Laxatives, Urine, Metabolites, LC-MS/MS, Positive ESI, Negative ESI, Qualitative

1. Introduction

Laxatives, both natural and synthetic, have been used for centuries by man at least dating back to the ancient Egyptians (1). Today, the medicinal objective of laxative use is to loosen stool and/or induce bowel movements for relief of constipation, medical procedures, or to ease the strain of defecation. There currently exist approximately seven classes of laxatives based on their pharmacological action. These classes include: stimulant laxatives, bulk-producing

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laxatives, emollients/lubricants, fecal softeners/surfactants, hyperosmotic agents, saline laxatives, anthraquinones, and a few miscellaneous substances (2). Certain foods are also well known to induce laxative-like effects, e.g., prunes, in a dose-related manner (3). A particular laxative class may be preferred to another based on the intended need. Nonmedical abuse of laxatives is well known in child abuse as well as in such conditions as eating disorders (4). Overuse of laxatives can lead to chronic diarrhea and other pathological states (5). Bio-monitoring of individuals for laxatives offers significant, relatively inexpensive assistance in addressing compliance, overuse, and abuse of such agents while potentially obviating the need for costly and significant medical tests.

The eight or so classes of laxatives comprise approximately 20 individual agents. In developing a bio-monitoring approach, it is beneficial to try to capture common metabolites of several compounds within different classes of laxative action. In respect to saline laxatives, the active agents are citrate, hydroxide, sulfate, and phosphate salts of magnesium and sodium. For these latter compounds, analysis of magnesium and/or sodium in stool or stool water may be worthwhile using flame emission spectroscopy or other suitable technique. For the other agents, urinary analysis for desacetylbisacodyl, emodin, aloe-emodin, and rhein captures a number of different substances within the laxative classes.

Ideally, a procedure for cleanup of urine prior to analysis would be simple with as few steps as possible. In respect to analytical finish, a sensitive and specific method would be preferred. One can find described methods for analysis of laxatives in biological matrices that employ thin-layer chromatography, high-performance liquid chromatography, and gas chromatography-mass spectrometry. Each of these methods has specific drawbacks, including lack of specificity, sensitivity, and/or the need for derivatization (4, 6, 7). The routine development and use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) in clinical and forensic toxicology has provided a sensitive and specific means for analysis of biological specimens for agents of interest, often with relatively less sample preparation time. While even LC-MS/MS has certain limitations and issues to be wary of, it adds to the armamentarium of analytical tools, especially for substances not readily amenable to other modes of analysis (8). For the previously described metabolites associated with laxative use, LC-MS/MS is a good candidate method, especially since deuterated internal standards are available for at least two of the analytes. Prior to analysis of urine, deconjugation of glucuronidated metabolites should take place. As three of the analytes of interest are anionic in nature (aloe-emodin, emodin, and rhein), the LC-MS/MS is run in both negative and positive ESI modes. The following described method is qualitative as quantitative results in urine cannot be correlated to dose.

2. Materials

Note: All reagents and solvents are of HPLC or analytical grade.

2.1. Prepared Reagents

1. 1.0 M sodium acetate buffer pH 5.5. Add 58 mL of glacial acetic acid to a 500 mL beaker containing approximately 300 mL of deionized water. Adjust pH to 5.5 with 50% aqueous sodium hydroxide. Transfer to a 1 L volumetric flask and dilute to volume with deionized water.
2. 0.50 M sodium acetate buffer pH 6.0. Add 30 mL of glacial acetic acid to a 500 mL beaker containing approximately 300 mL of deionized water. Adjust pH to 6.0 with 50% aqueous sodium hydroxide. Transfer to a 1 L volumetric flask and bring to volume with deionized water.
3. Deconjugation reagent: Dissolve 250,000 units of *Escherichia coli* β-*glucuronidase* Type IX-A, lyophilized powder in 25 mL of 0.50 M sodium acetate buffer pH 6.0. Aliquot and store at –20°C.
4. Mobile phase A (MP-A): 0.1% formic acid in deionized water. To 1 L of water, add 1 mL of 95–97% formic acid.
5. Mobile phase B (MP-B): 100% methanol.
6. Reconstitution solution: 80:20 (v/v%) water:methanol. To 80 mL water, add 20 mL of methanol and mix.

2.2. Standards and Controls

1. Desacetylbisacodyl, desacetylbisacodyl-d_{13}, rhein, emodin, emodin-d_4, and aloe-emodin (Toronto Research Chemicals, North York, Ontario, Canada) and phenolphthalein, phenolphthalein glucuronide (see Note 1), and biochanin A (Sigma, St. Louis, MO) were used to prepare standards, internal standards, and/or controls.
2. 100 μg/mL stock standards: Transfer 1.0 mg each of desacetylbisacodyl, aloe-emodin, emodin, rhein, and phenolphthalein into separate 10 mL volumetric flasks. Fill each to volume with methanol and dissolve. Store the stock standards at –10°C in amber glass bottles with Teflon-lined caps. Stock standards can be verified using UV spectrophotometry and Beer's Law (see Note 2).
3. Laxative mixed sub-stock standard: To a 10 mL volumetric flask, add 100 μL of each stock standard for desacetylbisacodyl, aloe-emodin, emodin, rhein, and phenolphthalein. Fill to volume with methanol. The final concentration of each compound in the mixed sub-stock is 1.0 μg/mL. Store this solution in a Teflon-lined capped amber vial at –10°C.

4. Laxative working standard: To a 13×100 mm tube containing 1 mL of blank urine, add 10 μL of laxative mixed sub-stock standard. Vortex to mix. Prepare fresh for each run; duplicate single point calibrators are run at the beginning and end of each batch.
5. Internal standard stock standards: Transfer 1.0 mg each of desacetylbisacodyl-d_{13}, emodin-d_4, and biochanin A (see Note 3) to separate 10 mL volumetric flasks. Dissolve and dilute to volume with methanol. Final concentration of each is 100 μg/mL.
6. Internal standard working solution: Transfer 1 mL of each stock solution into a 100 mL volumetric flask and bring to volume with isopropanol for a concentration of 1.0 μg/mL of each internal standard. Store this solution in a Teflon-lined capped amber vial at −10°C (see Note 4).
7. Quality control (QC) stock solutions: Prepare individual laxative stock QC solutions at 100 μg/mL as described above for the stock standards. Include phenolphthalein glucuroide in this stock solution. Store these solutions in Teflon-lined capped amber vials at −10°C.
8. QC sub-stock solutions: To independent 10 mL volumetric flasks, add 150 μL (high QC) or 50 μL (low QC) of each QC stock solution. Bring to volume with methanol. Concentrations are: high QC sub-stock, 1.5 μg/mL; low QC sub-stock, 0.5 μg/mL. Store these solutions in Teflon-lined capped amber vial at −10°C.
9. Working QC: Add 10 μL of the high and low QC sub-stocks to separate 13×100 mm tubes containing 1.0 mL of blank urine. Prepare fresh for each run. Concentrations are: high control, 15 ng/mL; low control, 5 ng/mL.

2.3. Analytical Materials

1. Instrument: Waters TQD API Tandem Mass Spectrometer with a Waters Acquity Ultra Performance LC equipped with Mass Lynx software.
2. Column: Hypersil Gold PFP 2.1 mm ID×100 mm 1.9 μm (Thermo Scientific) or equivalent.

3. Methods

3.1. Sample Preparation

Due to the use of chemicals and material of biological origin, procedures consistent with a laboratory's Chemical Hygiene and Bloodborne Pathogens standard operating procedures must be followed.

1. Transfer 1.0 mL of blank urine, standards, controls, and patient specimens to appropriately labeled 13×100 mm tubes. Duplicate single point calibrators are run in the beginning and end of each batch.
2. Add 100 µL of working internal standard solution to each tube; vortex to mix.
3. Add 0.5 mL of 1.0 M acetate buffer pH 5.5 to each tube; vortex to mix (see Note 5).
4. Add 10 µL of *E. coli* (10,000 units/mL β-glucuronidase) solution, cap and incubate for 3 h at 37°C. Following hydrolysis, specimens are cooled to room temperature and mixed.
5. Add 3.0 mL of *n*-butyl chloride to each tube; cap and rotomix for 15 min (see Note 6).
6. Centrifuge for approximately 10 min at approximately 2,300 RCF.
7. Transfer upper organic layer to appropriately labeled 12×75 mm tubes. This can be accomplished by using pipettes or by freezing the bottom layer in an acetone-dry ice bath and decanting the upper layer.
8. Evaporate to dryness at 55°C±5°C under nitrogen.
9. Reconstitute with 200 µL of 80:20 water:methanol.
10. Vortex briefly, and transfer to autosampler vials (see Note 7). Extracts are ready for LC-MS/MS analysis.

3.2. Analysis (see Note 8)

A representative chromatogram is shown in Fig. 1.

1. Injection volume: 20 µL.
2. Mobile phase gradient.

   Initial: 5% MP-B.

   0–1 min: linear gradient to 70% MP-B.

   1–2 min: hold at 70% MP-B.

   2–3 min: linear gradient to 95% MP-B.

   3–4 min: hold at 95% MP-B.

   4 min: return to 5% MP-B.
3. Mass transitions are shown in Table 1.
4. Instrument parameters are shown in Table 2.
5. Scan functions are shown in Table 3.

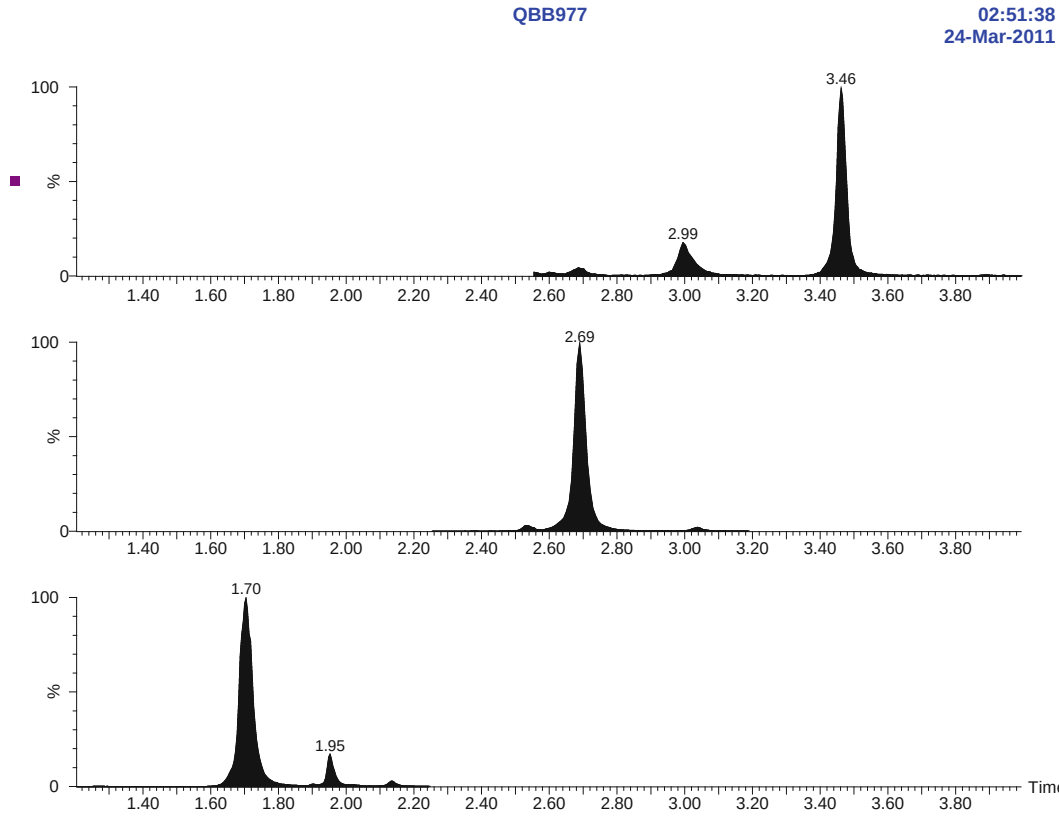

Fig. 1. MRM data for the amphetamines analysis by LC-MS/MS. Sample total ion chromatograms. 1.70 min, desacetyl-bisacodyl; 1.95 min, phenolphthalein; 2.58 min, aloe-emodin; 2.69 min, biochanin A; 2.99 min, rhein; 3.46 min, emodin.

Table 1
Transitions monitored

Analyte	Quant ion	Ratio ion
Desacetyl bisacodyl-d_{13}	291 > 193	291 > 176
Desacetyl bisacodyl	278 > 184	278 > 167
Phenolphthalein	319 > 225	319 > 197.3
Biochanin A	283 > 268	283 > 211
Aloe-emodin	269.1 > 240.1	269.1 > 211.2
Emodin-d_4	273.1 > 229.5	273.1 > 245.2
Emodin	269.1 > 225.2	269.1 > 241.1
Rhein	283 > 239	283 > 183

Table 2
Instrument parameters

Parameter	Function 1	Function 2	Function 3
Polarity	ES+	ES-	ES-
Calibration	Static 2	Static 2	Static 2
Capillary (kV)	3.00	3.50	3.50
Cone (V)	40.00	35.00	35.00
Extractor (V)	3.00	3.00	3.00
RF (V)	0.10	0.10	0.10
Source temperature (°C)	150	150	150
Desolvation temperature (°C)	450	450	450
Cone gas flow (L/h)	35	35	35
Desolvation gas flow (L/h)	900	800	800
Collison gas flow (mL/min)	0.20	0.35	0.35
LM 1 resolution	15.00	10.00	10.00
HM 1 resolution	15.00	10.00	10.00
Ion energy 1	0.50	0.50	0.50
MS mode entrance	50.00	50.00	50.00
MS mode collision energy	4.00	2.00	2.00
MS mode exit	50.00	50.00	50.00
MSMS mode entrance	0.00	0.00	0.00
MSMS mode collision energy	30.00	25.00	25.00
MSMS mode exit	1.00	1.00	1.00
LM 2 resolution	15.00	10.00	10.00
HM 2 resolution	15.00	10.00	10.00
Ion energy 2	2.00	2.00	2.00
Gain	1.00	1.00	1.00
Multiplier	–482.11	–482.11	–482.11
Active reservoir	B	B	B
Autotune reservoir	B	B	B

Table 3
Scan functions

Channel	Reaction	Dwell (s)	Cone (V)	Collision energy	Delay (s)	Compound	Comments
Function 1. 1.2–2.25 min (positive ion mode)							
1	278.00 > 167.00	0.040	40.0	35.0	0.020	Desacetyl-bisacodyl	Positive ion
2	278.00 > 184.00	0.040	40.0	25.0	0.010	Desacetyl-bisacodyl	Positive ion
3	291.00 > 176.00	0.040	40.0	35.0	0.010	Desacetyl-bisacodyl-D13	Positive ion
4	291.00 > 193.00	0.040	40.0	25.0	0.010	Desacetyl-bisacodyl-D13	Positive ion
5	319.00 > 197.30	0.040	40.0	35.0	0.010	Phenolphthalein	
6	319.00 > 225.00	0.040	40.0	25.0	0.010	Phenolphthalein	
Function 2. 2.25–3.2 min (negative ion mode)							
1	269.00 > 211.20	0.070	50.0	42.0	0.020	Aloe-Emodin	
2	269.00 > 240.00	0.070	50.0	22.0	0.010	Aloe-Emodin	
3	283.00 > 211.00	0.020	45.0	40.0	0.010	Biochanin A	
4	283.00 > 268.00	0.020	45.0	25.0	0.010	Biochanin A	
Function 3. 3.2–4 min (negative ion mode)							
1	269.10 > 225.00	0.040	55.0	26.0	0.020	Emodin	
2	269.10 > 241.10	0.040	55.0	30.0	0.010	Emodin	
3	273.10 > 229.50	0.040	55.0	26.0	0.010	Emodin-D_4	
4	273.10 > 245.20	0.040	55.0	30.0	0.010	Emodin-D_4	
5	283.00 > 183.00	0.040	30.0	30.0	0.010	Rhein	
6	283.00 > 239.00	0.040	30.0	15.0	0.010	Rhein	

4. Notes

1. Phenolphthalein glucuronide is used as an enzymatic hydrolysis control.
2. The use of Beer's Law can be used to determine the concentration of a solution via use of the molar absorptivity constant (ε). Classically, the following formula is applied:

$$C = A / \varepsilon b,$$

where C = concentration,

A = absorbance,

ε = molar absorptivity coefficient,

b = path length.

Molar absorptivity constants for various substances in a given solution at a given concentration can be found in many references, e.g., Merck Index.

3. Biochanin A is used as an analog internal standard for rhein and aloe-emodin.
4. Isopropanol is used to minimize emulsion formation during the extraction process.
5. Extraction solvent and pH were chosen to minimize ion suppression of rhein and aloe-emodin. The extraction efficiency is not optimal under these conditions.
6. Rotomix rate is approximately 30 rpm.
7. Validation studies have shown samples are stable in autosampler vials for no longer than 24 h; samples must be injected within 24 h of preparation.
8. Each analyte must meet relative retention time parameters (±2% of calibrators) and ratio characteristics (±30% of calibrators). Relative retention time is defined as the retention time of the analyte divided by the retention time of the internal standard. The ratio characteristic is defined as the ratio of the response of one transition of the analyte of interest to that of another transition for that analyte. This ratio should be relatively constant from sample to sample. The matrix blank response must be no greater than 25% of the lowest calibrator.

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Chapter 15

Simultaneous Determination of Cyclosporine, Sirolimus, and Tacrolimus in Whole Blood Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

A multiple reaction monitoring, positive ionization electrospray, liquid chromatography–tandem mass spectrometry (LC-MS/MS) method is described for the simultaneous quantification of cyclosporine, sirolimus, and tacrolimus in human whole blood. Proteins in the samples are precipitated with a mixture of methanol and zinc sulfate. The supernatant is injected into the LC-MS/MS for analysis. Chromatography involves the use of a C18 column and ammonium acetate/water/methanol-containing mobile phases. The MS/MS is operated in positive ion electrospray mode. Quantification is achieved by comparing peak area ratios of MRMs of analytes and internal standards with that of calibrators. Calibration curves are constructed from peak area ratios of MRMs of calibrators and internal standards versus concentrations. MRMs used are ascomycin (m/z 809.5 → 756.5), cyclosporine A (m/z 1,219.9 → 1,203.1), cyclosporine D (m/z 1,234.0 → 1,217.0), sirolimus (m/z 931.6 → 864.5), and tacrolimus (m/z 821.5 → 768.4).

Key words: Cyclosporine, Sirolimus, Tacrolimus, Mass spectrometry, Therapeutic drug monitoring

1. Introduction

Cyclosporine A (CSA), sirolimus, and tacrolimus are commonly used immunosuppressive drugs. Cyclosporine and tacrolimus are calcineurin inhibitors, whereas sirolimus is a mammalian target of rapamycin (mTOR) inhibitor. Calcineurin inhibitors bind to immunophilins, and drug-immunophilin complexes inhibit calcineurin activity, which in turn prevents nuclear translocation of the nuclear factor of activated T cells (NFAT) (1, 2). This results in inhibition of activation and proliferation of CD4 and CD8 lymphocytes by inhibiting IL-2 production. The mTOR protein is a

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specific cell regulator and its inhibition results in suppression of cytokine-driven T lymphocyte proliferation (1, 3).

Since these drugs significantly accumulate in red blood cells, whole blood is the specimen of choice for the determination of CSA, sirolimus and tacrolimus. In general, trough concentrations are monitored for therapeutic drug monitoring of immunosuppressants (3). However, in recent years, peak concentrations of CSA have been correlated with clinical outcome and many studies have found that peak levels (samples drawn 2 h after an oral dose, called C2 monitoring) correlate better with the total drug exposure and clinical outcome as compared to the trough levels (4–6).

The commonly used methods for the determination of these drugs are immunoassays and chromatography. Most immunoassays for immunosuppressants are semiautomated since extraction of drugs from the whole blood is needed before analysis. Immunoassays are convenient due to automation, but have problems with cross-reactivity with drug metabolites (3, 6). Both polyclonal and monoclonal antibody-based assays are available. Monoclonal antibody-based immunoassays are more specific. HPLC with ultraviolet detection and tandem mass spectrometry are commonly used chromatographic methods for the assay of immunosuppressants. Due to their specificity and sensitivity, tandem mass spectrometry assays are preferred and are now in wide use (7, 8). The other major advantage of tandem mass spectrometry assays is their ability to simultaneously measure several immunosuppressants (7–10). Pharmacokinetic properties of CSA, sirolimus, and tacrolimus are shown in Table 1 (3, 6, 11).

Table 1
Pharmacokinetic properties of immunosuppressants

Drug	Half-life (h)	V_d (L/kg)	Oral bioavailability (%)	Protein binding (%)	Therapeutic range (ng/mL)
Cyclosporine A	6–24	4–6	30–90	95	Kidney: 100–200; BMT: 100–250; Heart: 100–200; Liver: 100–400
Sirolimus	8–20	4–20	14–18	90	5–20
Tacrolimus	8–16	0.3–2.6	7–55	95	5–20

The therapeutic ranges of immunosuppressants vary with methods and are generally lower after the transplant is established

2. Materials

2.1. Samples

Whole blood collected in EDTA containing tubes. Samples are stable for 1 week when stored at 2–8°C.

2.2. Internal Standards

1. Primary stock cyclosporine D solution, 1 mg/mL (Toronto Research, North York, Canada). Prepare working internal standard solution by diluting stock solution 4× in methanol, to a concentration of 250 μg/mL. The solution is stable for 1 year at −20°C.
2. Primary stock ascomycin solution, 1 mg/mL (Toronto Research, North York, Canada). Prepare working internal standard solution by diluting stock solution 40× in methanol, to a final concentration of 25 μg/mL. The solution is stable for 1 year at −20°C.

2.3. Reagents and Buffers

1. 7.5 M Ammonium acetate. Purchased as solution (Sigma-Aldrich, St. Louis, MO).
2. 0.3 mM Zinc sulfate. Purchased as solution (Sigma-Aldrich, St. Louis, MO).
3. Precipitating reagent: Mix 70 mL methanol and 30 mL 0.3 mM zinc sulfate. Add 20 μL of working cyclosporine D internal standard and 20 μL of working ascomycin internal standard. This provides cyclosporine D and ascomycin concentrations of 50 ng/mL and 5 ng/mL, respectively.
4. Mobile phase A: 20 mM ammonium acetate in water. Add 2.7 mL of 7.5 mM ammonium acetate to a 1 L volumetric flask. Fill to the mark with HPLC grade water. Mix and degas. Store at room temperature. Stable for 1 month.
5. Mobile phase B: 20 mM ammonium acetate in methanol. Add 2.7 mL of 7.5 mM ammonium acetate to a 1 L volumetric flask. Fill to the mark with methanol. Mix and degas the mixture and store at room temperature. Stable for 1 month.

2.4. Standards and Calibrators

1. Primary standards–CSA, sirolimus, tacrolimus, 1 mg/mL each, in acetonitrile (Cerilliant, Round Rock. TX).
2. Stock standards: Add 1 mL each of 1 mg/mL primary standards of CSA, sirolimus, and tacrolimus to separate 10 mL volumetric flasks and fill to the mark with methanol. This provides the final concentration of 100 μg/mL for each standard.
3. Working standards mixture: Add 1.25 mL CSA, 125 μL sirolimus and 125 μL tacrolimus stock standards to a 250 mL volumetric flask, and bringing the volume to the mark with drug-free whole blood. Final concentrations of CSA, sirolimus, and tacrolimus are 500, 50, and 50 ng/mL, respectively.
4. Prepare the calibrators according to Table 2 (see Note 1). Aliquot and freeze. Stable for 1 year at −20°C.

Table 2
Preparation of CSA, sirolimus, and tacrolimus calibrators

Calibrator	Drug free whole blood (mL)	Working mixture (mL)	Final concentrations (ng/mL)		
			CSA	Sirolimus	Tacrolimus
1	100	0	0	0	0
2	96	4	20	2	2
3	90	10	50	5	5
4	80	20	100	10	10
5	50	50	250	25	25
6	0	100	500	50	50

2.5. Quality Control Samples

1. Bio-Rad Lyphochek® (Irvine, CA) whole blood immunosuppressant controls (five levels) are used. The controls are reconstituted with deionized water. Reconstituted controls are stable for 14 days.

2.6. Supplies

1. Supelcosil LC-18, 5 cm × 4.6 mm × 3 μm, analytical column (Sigma-Aldrich, St. Louis, MO).
2. Guard column: Pinnacle II, C18,10 mm × 4 mm × 5 μm (Restek, Bellefonte, PA).

2.7. Equipment

1. AB Sciex LC-MS/MS 4000Q TRAP (Foster City, CA).
2. Shimadzu Prominence HPLC system with auto sampler, two pumps, and degasser (Lenexa, KS).

3. Methods

3.1. Stepwise Procedure

1. Vortex and mix well all samples, controls, and calibrators.
2. Pipette 100 μL of samples, controls, and calibrators to microcentrifuge tubes.
3. Add 500 μL of precipitating reagent containing internal standards to each tube.
4. Immediately cap the samples and vortex for 10–15 s (see Note 2).
5. Rock the tubes for 10 min.
6. Centrifuge the tubes at 10,000 × *g* for 5 min.
7. Using disposable tips, transfer 200 μL of supernatants into autosampler vials (see Note 3).
8. Inject 20 μL into liquid chromatography–tandem mass spectrometry (LC-MS/MS) for analysis.

3.2. Instrument Operating Conditions

1. HPLC parameters: injection volume: 20 µL, flow rate: 1.00 mL/min, column temperature: 65°C
2. Mobile phase gradient
   0–0.2 min: hold at 80% MP-B.
   0.2–2 min: linear gradient to 100% MP-B.
   2–2.5 min: linear gradient to 80% MP-B.
   2.5–2.6 min: hold at 80% MP-B.
3. MS/MS parameters are given in Table 3.
4. MRMs are given in Table 4.

Table 3
MS-MS parameters

Nebulizer, curtain, collision gas	Nitrogen
Curtain gas	25 psi
Nebulizer gas	60 psi
Turbo flow	55 psi
Turbo ion spray temperature	350°C
Collision gas	Medium
Declustering potential (V)	Ascomycin–76, CSA–73, CSD–73, Sirolimus–61 and Tacrolimus–81
Entrance potential (V)	10
Exit potential (V)	Ascomycin–20, CSA–12, CSD–12, Sirolimus–24 and Tacrolimus–20
Collision energy (eV)	Ascomycin–29, CSA–30, CSD–27, Sirolimus–25 and Tacrolimus–29

Table 4
MRMs for ascomycin, cyclosporine A, cyclosporine D, sirolimus and tacrolimus

Analyte	Q1	Q3
Ascomycin	809.5	756.5
Cyclosporine A	1,219.9	1,203.1
Cyclosporine D	1,234.0	1,217.0
Sirolimus	931.6	864.5
Tacrolimus	821.5	768.4

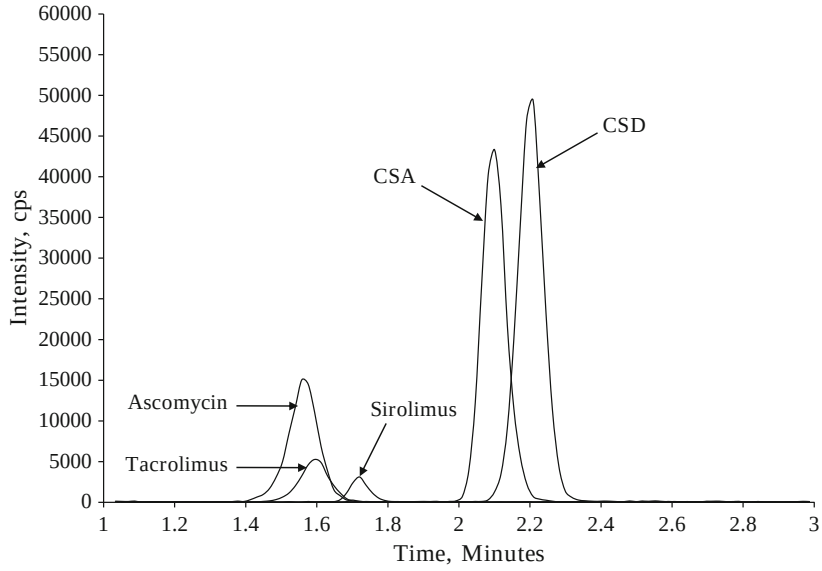

Fig. 1. A representative HPLC/MS/MS chromatogram for ascomycin, CSA, cyclosporine D, sirolimus and tacrolimus.

3.3. Data Analysis

1. A representative LC-MS/MS chromatogram is shown in Fig. 1.
2. Data are collected and analyzed using Analyst 1.5.1 software (AB Sciex, Foster City, CA).
3. Peak area ratios of MRMs of analytes and internal standards are used for quantification. Cyclosporine D is used as internal standard for quantification of CSA and ascomycin is used as internal standard for the quantification of sirolimus and tacrolimus.
4. Calibration curves are constructed from peak area ratios of MRMs of calibrators and internal standards versus concentrations.
5. A typical calibration curve has a correlation coefficient (r^2) > 0.99.
6. Typical intra- and inter-assay imprecision is <10%.
7. Quality control samples are evaluated with each run. The run is considered acceptable if calculated concentrations of controls are within ± 20% of target values.
8. Samples with results greater than upper limit of linearity should be diluted with negative whole blood and reextracted.
9. Samples with results less than lower limit of linearity are reported as less than the lowest calibrator.

4. Notes

1. If peak concentrations (C2) of cyclosporine are measured, an additional CSA calibrator with a concentration of 1,500 ng/mL is also used. Like other calibrators, it is prepared in whole blood using stock cyclosporine standard.
2. The samples should be homogenous; if not, vortex the samples further. Sometimes tapping tubes on the countertop may be needed to dislodge the precipitate pellet.
3. Make sure there are no bubbles or particulate matter. Tap the vials with fingers to remove air bubbles. If the particulate matter is present, centrifuge the extract again to remove the particulate matter.

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Chapter 16

Quantification of Tricyclic Antidepressants Using UPLC-MS/MS

Kamisha L. Johnson-Davis, JoEtta M. Juenke, Rebecka Davis, and Gwendolyn A. McMillin

Abstract

Depression is a psychiatric condition that affects about 120 million people worldwide and can interfere with independence and productivity in essentially all aspects of daily life. Depression is also associated with risk of self-harm, and ultimately suicide. Antidepressant medications are widely used to treat symptoms of depression. While there are several classes of antidepressants, therapeutic drug management (TDM) is most common for the tricyclic antidepressants (TCAs). TDM of TCAs is important due to wide inter-individual variability in pharmacokinetics, production of active metabolites, and a high risk of drug–drug interactions. In addition, TDM of some TCAs can be used to optimize dose, wherein concentration relationships are recognized for both therapeutic response and potentially life-threatening toxicity. In many clinical scenarios, TDM of TCAs is accomplished by currently available point of care or automated immunoassays that provide a "total" TCA concentration. However, these assays may not be adequately specific to meet the needs of all clinical scenarios, and hence, chromatographic separation and quantification of individual TCA parent drugs and active metabolites that may contribute to the "total" TCA concentration is sometimes required. This chapter describes an analytical method designed to detect and/or quantify clinically significant concentrations of nine TCAs (amitriptyline, nortriptyline, imipramine, desipramine, doxepin, nordoxepin, protriptyline, clomipramine, and norclomipramine) in serum or plasma, using ultra pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The sample preparation employs a rapid protein precipitation with 50:50 MeOH:acetonitrile, high speed centrifugation, and injection of 5 μL of supernatant onto the instrument, with a 5 min run-time.

Key words: Tricyclic antidepressants, TCAs, Ultra pressure liquid chromatography-tandem mass spectrometry, UPLC-MS/MS, Therapeutic drug monitoring, TDM, Waters

1. Introduction

Depression and anxiety disorders are common forms of mental illness. According to the World Health Organization, depression affects approximately 120 million people worldwide. In 2008, the National Institute on Mental Health (NIMH) reported that

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~17 million adults in the United States are diagnosed with depression each year. Although depression can be treated, many individuals are unaware of their condition and do not seek help. Of those individuals that are diagnosed, ~50% receive therapy; however, only ~20% receive proper therapy that is in agreement the American Psychological Association guidelines. Clinical depression is characterized by persistent feelings of sadness and symptoms such as fatigue, overeating or loss of appetite, disinterest in pleasurable activities, agitation or hostility, and suicidal thoughts. Depression can therefore interfere with and adversely affect all aspects of daily life (1, 2).

Antidepressant medications are the preferred therapy to manage symptoms of moderate to severe depression. However, a large percentage of patients do not respond to antidepressant therapy, resulting in therapeutic failure in 40–60% of individuals (3). For example, only 60% of patients that receive monotherapy for antidepressant drugs respond well to treatment; whereas, the percentage is increased to 80% for individuals on multidrug therapy (4). Although the specific mechanisms of action are not fully elucidated, antidepressant drugs are thought to function primarily by modulating monoamine receptors and neurotransmitters such as serotonin, norepinephrine, and dopamine in the brain (5). Consequently, many classes of antidepressant drugs are named after the best recognized mechanism of action for that drug. Currently available classes of antidepressants include selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and escitalopram; serotonin-norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine and duloxetine; dopamine reuptake blockers such as bupropion; monoamine oxidase inhibitors (MAOIs) such as isocarboxazid; noradrenergic antagonists such as mirtazapine; serotonin receptor antagonists such as trazodone; tetracyclic compounds such as maprotiline; and tricyclic antidepressants (TCAs) such as amitriptyline and doxepin. Based on these mechanisms of action, it is not surprising that antidepressants are also used to manage other clinical conditions related to neurotransmitter imbalances, such as neuropathic pain, attention deficit hyperactivity disorder, and migraine headaches.

Therapeutic drug management (TDM) of most antidepressants is not widely available or widely utilized, for any clinical indication. The primary benefits of TDM in the management of antidepressant therapy could include assessment of patient compliance, detection of drug–drug interactions or altered pharmacokinetics, and evaluating the concentration relationship of individual patient response and/or thresholds for dose-related side effects. Of all the classes of antidepressant drugs, TDM of TCAs is most common.

TCAs are named as such because of their core three-ring structure (Fig. 1). Common TCA drugs used clinically today include amitriptyline, nortriptyline, clomipramine, imipramine, desipramine, protriptyline, and doxepin. TCAs are metabolized primarily by the cytochrome P450 (CYP) 2D6 liver enzyme, which is known

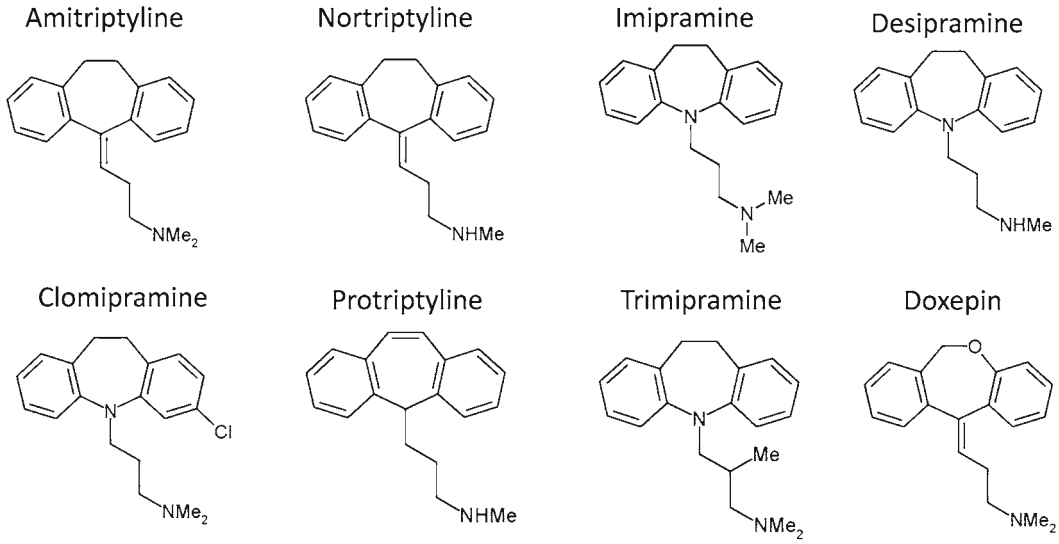

Fig. 1. Structure of tricyclic antidepressants.

to be polymorphic and contribute to inter-patient variability in plasma concentrations of both parent and metabolite drugs. TCA metabolism may involve other CYP isozymes, particularly 2C19 and 3A4. Therapeutic efficacy of TCAs is thereby affected by many co-administered drugs or supplements that induce or inhibit related CYP-mediated metabolism.

Therapeutic ranges utilized for TDM of TCAs are based on pre-dose (trough) plasma collections, and are best interpreted after several weeks of constant dosing, when signs of patient response and steady state concentrations are anticipated. TCAs that exhibit a good correlation between plasma concentration and therapeutic effect include amitriptyline, desipramine, imipramine, and nortriptyline (6). The therapeutic range varies with each compound (Table 1), and whether an active drug is produced by metabolism. Note that some active metabolites are available independently as "parent" drugs. TCAs with active metabolites (in parentheses) include: amitriptyline (nortriptyline), imipramine (desipramine), clomipramine (norclomipramine), and doxepin (nordoxepin). The parent drug and active metabolite, if applicable, should both be quantified and the sum considered relative to the proper interpretation of TDM.

Currently available immunoassays designed to detect and quantify TCAs provide a "total" concentration of cross-reacting substances. This total concentration may be adequate for drugs and equipotent metabolites that exhibit comparable cross-reactivity with the detection antibody upon which the immunoassay is based, especially when the total concentrations are consistent with clinical expectations. Immunoassay-based results are less useful for evaluating compounds of differential cross-reactivity and potency, wherein falsely elevated or falsely low concentrations may be reflected by a "total." Falsely elevated immunoassay-based results are likely when non-TCA

Table 1
TCA pharmacokinetics

Compound (metabolite)	Half-life (hours)	Time to peak (hours)	Therapeutic range (ng/mL)	Toxic threshold (ng/mL)
Amitriptyline (Nortriptyline)	8–51	2–4	Total (Ami. + Nor.) 95–250	Total: >500
Nortriptyline	15–90	5.3	50–150	>500
Imipramine (Desipramine)	6–20	4	Total (Imip. + Desip.) 150–300	Total: >500
Desipramine	12–54	3–6	100–300	>500
Doxepin (Nordoxepin)	8–25	2	Total (Dox. + Nordox.) 100–300	Total: >400
Protriptyline	54–92	6–12	70–240	>400
Clomipramine (Norclomipramine)	12–36	4	Total (Clom. + Norclom.) 220–500	Total: >900

cross-reacting compounds (e.g., carbamazepine) are present. Falsely low or high TCA results may contribute to an inappropriate dose adjustment or unnecessary clinical testing. Immunoassays also cannot identify or characterize metabolic variability, such as is observed with inherited variation in CYP genes, variation in concentration of CYPs, or with drug–drug interactions.

Toxicity with TCAs can be life threatening, and can occur when plasma concentrations exceed 500 ng/mL. Indeed, adverse effects associated with excessive TCA concentrations include cardiac toxicity (7) and anticholinergic effects like dry mouth, constipation, urinary retention, decreased sweating, and hyperthermia. While an immunoassay for TCA quantification may assist with detection of toxic total concentrations, investigation of TCA-associated toxicity, pharmacokinetic variability, or other results inconsistent with clinical suspicions may require a technology that provides better specificity than an immunoassay. Only a chromatographic assay can independently detect and quantify TCAs and active metabolites such that pharmacokinetic variation or polypharmacy can be identified. Here a chromatographic technique, with mass spectrometric detection, is described.

2. Materials

2.1. Reagents

1. All drug stock standards are obtained from Cerilliant (Round Rock, Texas) at a concentration of 1 μg/μL, prepared in methanol (MeOH): amitriptyline, doxepin, clomipramine,

imipramine, norclomipramine, nortriptyline, nordoxepin, desipramine, protriptyline.

2. All stock internal standards are obtained from Cerilliant at a concentration of 0.1 μg/μL, prepared in MeOH: doxepin-D_3, clomipramine-D_3, imipramine-D_3, nortriptyline-D_3, desipramine-D_3, protriptyline-D_3.
3. Quality controls are obtained from UTAK Laboratories and contain doxepin, nordoxpin, imipramine, desipramine, amitriptyline, nortriptyline, protripyline, clomipramine, and norclomipramine.

2.2. Combined Stocks for Instrument Prime Solution

1. Combined analyte stocks: 10 ng/μL doxepin, nordoxepin, imipramine, desipramine, amitriptyline, nortriptyline, and protriptyline, and 20 ng/μL clomipramine and norclomipraine, stored in amber-tinted glass. For example, add 890 μL methanol to a new amber vial. Add 10 μL each of doxepin, nordoxepin, imipramine, desipramine, amitriptyline, nortriptyline, and protriptyline. Add 20 μL each of clomipramine and norclomipramine.
2. Combined internal standard (IS) stocks: 10 ng/μL doxepin-D_3, imipramine-D_3, desipramine-D_3, nortriptyline-D_3 and protriptyline-D_3 and 20 ng/μL clomipramine-D_3, stored in amber-tinted glass. For example, add 300 μL methanol to a new amber vial. Add 100 μL each of doxepin-D_3, imipramine-D_3, desipramine-D_3, nortriptyline-D_3, and protriptyline-D_3, and 200 μL clomipramine-D_3.

2.3. Standards and Controls

1. 0.4/0.8 ng/μL Working standard: 0.4 ng/μL doxepin, nordoxepin, imipramine, desipramine, amitriptyline, nortriptyline, and protriptyline, and 0.8 ng/μL clomipramine and norclomipramine.
   (a) From individual stocks: Add 5 mL methanol to a 10 mL volumetric flask. Add 4 μL each of doxepin, nordoxepin, imipramine, desipramine, amitriptyline, nortriptyline, and protriptyline stock standards. Add 8 μL each of clomipramine and norclomipramine stock standards. Fill to volume with methanol and mix.
   (b) From combined analyte stock: Add 5 mL methanol to a 10 mL volumetric flask. Add 400 μL of the combined analyte stock and fill to volume with methanol. Mix well.
2. Low and High Controls (A & B)
   (a) UTAK controls Levels 1 and 2. Add 5 mL of Type 1 water to lyophilized vial, mix, and equilibrate overnight. Stable after reconstitution for 25 days at 2–8°C. Target concentrations are: Level 1, 150 ng/mL clomipramine and norclomipramine, 75 ng/mL all other compounds; Level 2, 300 ng/mL clomipramine and norclomipramine, 250 ng/mL all other compounds.

2.4. Prepared Solutions

1. Precipitation solution: 50:50 methanol:acetonitrile with internal standard, 37.5 ng/mL in doxepin-D_3, imipramine-D_3, desipramine-D_3, nortriptyline-D_3, and protriptyline-D_3, and 75 ng/mL in clomipramine-D_3. Fill a 500 mL volumetric flask halfway with 50:50 methanol:acetonitrile. Add 187.5 µL each of doxepin-D_3, imipramine-D_3, desipramine-D_3, nortriptyline-D_3, and protriptyline-D_3. Add 375 µL of clomipramine-D_3. Fill to the mark with 50:50 methanol:acetonitrile. Mix well.
2. Prime solution: 6.7 ng/mL of doxepin, nordoxepin, imipramine, desipramine, amitriptyline, nortriptyline, and protriptyline, 13.3 ng/mL of clomipramine and norclomipramine, 25 ng/mL of doxepin-D_3, imipramine-D_3, desipramine-D_3, nortriptyline-D_3, and protriptyline-D_3, and 50 ng/mL of clomipramine-D_3. Add 10 mL methanol to a new 16×100 screw cap tube. Add 6.7 µL of the combined standard stock and 25 µL of the combined internal standard stock. Mix well.
3. Mobile phase A: 0.1% formic acid in water. Add 1 mL formic acid for each 1 L of Type 1 water. Store at room temperature. Note: use appropriate personal protective equipment and precautions when handling formic acid.
4. Mobile phase B: 0.1% formic acid in acetonitrile. To a 4 L bottle of HPLC grade acetonitrile, add 4 mL formic acid. Store at room temperature. Note: use appropriate personal protective equipment and precautions when handling formic acid.
5. Calibrators: Calibrators are prepared fresh for each run by spiking 0.1 mL blank plasma using the appropriate amount of 0.4/0.8 ng/µL working standard as follows: Cal 1, 5 µL working standard; Cal 2, 25 µL working standard; Cal 3, 50 µL working standard; Cal 4, 75 µL working standard. Concentrations for calibrators 1–4 are 40, 200, 400, and 600 ng/mL, respectively, for clomipramine and norclomipramine, and 20, 100, 200, and 300 ng/mL, respectively, for all other compounds.

3. Methods

3.1. Sample Preparation

1. Add 100 µL control and patient plasma or serum to microcentrifuge tubes (see Note 1).
2. Add appropriate working standard spike and 100 µL blank plasma to prepare the four calibrators as described above. A total ion chromatogram obtained with Cal 4 is shown in Fig. 2.
3. Add 200 µL of the precipitation solution/internal standard to each tube.

4. Cap tubes and invert rack to mix.
5. Centrifuge for 5 min at 20,817 ×*g* and transfer the supernatant into autosampler vials.
6. Perform priming injections (see Note 2).
7. Inject 2–10 µL of each sample into the UPLC-MS/MS.

3.2. UPLC Conditions

The LC-MS/MS is a Waters Acquity UPLC TQD with associated Waters Masslynx software—This assay utilizes electrospray ionization, multiple reaction monitoring (MRM), and positive ion mode. The data analysis is performed using the Waters Quanlynx software (see Note 3).

1. Column: Acquity UPLC HSS T3 1.8 um, 2.1×50 mm (Waters).
2. Column temperature: room temperature (26±5°C).
3. LC flow: 0.60 mL/min.
4. LC injection volume: 2–10 µL (see Note 4).
5. Mobile phase gradient.

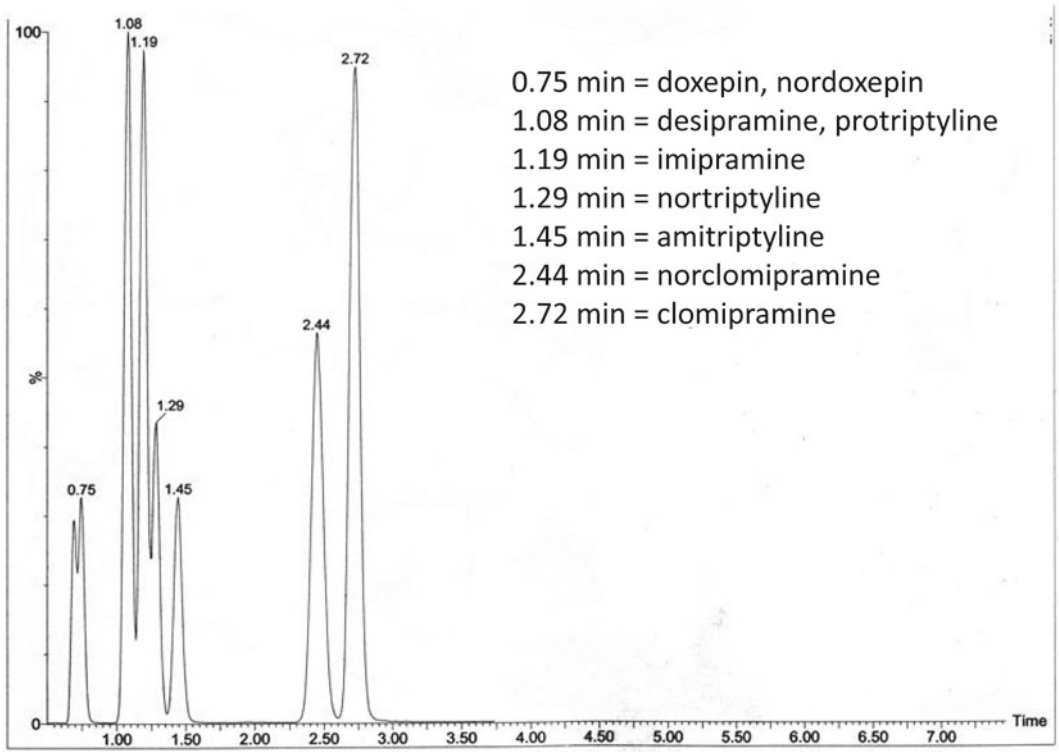

Fig. 2. Total ion chromatogram of Cal 4. The retention times demonstrate co-elution of some compounds. Deuterated internal standards elute at the same time as the non-deuterated analog. All compounds are detected based on multiple reaction monitoring (see Table 2).

0–2 min: hold at 30% MP-B.

2–3.5 min: linear gradient to 40% MP-B.

3.5–4 min: 95% MP-B.

4–4.5 min: 30% MP-B.

3.3. MS Conditions

1. ES + source:
   (a) Capillary: 3.00 kV.
   (b) Extractor: 3.00 V.
   (c) RF: 0.1 V.
   (d) Source temperature: 120°C.
   (e) Desolvation temperature: 350°C.
   (f) Cone gas flow: 25 L/h.
   (g) Desolvation gas flow: 900 L/h.
   (h) Collision gas flow: 0.10 mL/min.
2. Analyzer:
   (a) LM 1 Resolution: 13.5.
   (b) HM 1 Resolution: 13.5.
   (c) Ion energy 1: 0.1.
   (d) MSMS mode entrance: 1.00.
   (e) MSMS mode exit: 0.50.
   (f) LM 2 resolution: 13.5.
   (g) HM 2 resolution: 13.5.
   (h) Ion energy 2: 0.9.
   (i) MRM transitions are shown in Table 2. Each analyte is listed under the internal standard that it is referenced to. Quantifying transition is listed first (parent ion), followed by the qualifying transition (daughter ion). Interferences are detailed in Note 5.

4. Notes

1. Controls should be assayed with each analytical run and should fall within ±2 standard deviations of the mean.
2. Prior to the analytical run, two prime injections are done followed by two methanol blank injections. This decreases shifting in the retention times and primes the column.
3. Calculations are performed by Quanlynx, using peak area counts, and are important for quantification. The first transition (Multiple Reaction Monitoring 1 or MRM1) listed for each

Table 2
Mass transitions and parameters

Analyte	Retention Time (min)	Parent ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Doxepin-D_3	0.75	283.15	107.00 235.10	30 30	25 15
Doxepin	0.75	280.10	107.05 235.10	30 30	25 15
Nordoxepin	0.70	266.10	107.05 235.10	30 30	20 15
Nortriptyline-D_3	1.29	267.15	91.00 233.10	30 30	20 15
Nortriptyline	1.29	264.10	91.05 233.10	30 30	20 15
Amitriptyline	1.45	278.20	91.00 105.00	35 35	25 25
Imipramine-D_3	1.19	284.20	89.10 61.10	30 30	15 30
Imipramine	1.19	281.15	86.05 58.10	30 30	15 30
Desipramine-D_3	1.07	270.15	75.05 47.10	25 25	15 30
Desipramine	1.07	267.15	72.05 44.10	30 25	20 30
Protriptyline-D_3	1.09	267.20	155.10 191.10	35 35	20 25
Protriptyline	1.09	264.20	155.05 191.10	35 35	20 25
Clomipramine-D_3	2.72	318.15	89.10 61.10	30 30	20 30
Clomipramine	2.72	315.10	86.05 58.05	30 30	20 30
Norclomipramine	2.44	301.10	72.05 44.05	30 30	15 35

The product ion used for quantitation is listed above the qualifying product ion. Analytes are listed below the appropriate internal standard

analyte in Table 2 was assigned as the quantifying transition and the second one (MRM 2) is the qualifying transition.

(a) Qualifying ion ratios. Qualifying ion ratios for each analyte and internal standard are determined by the ratio of MRM 1/MRM 2. The qualifying ion ratio for each analyte should fall within 25% of the average of the calibrators.

(b) Quantifying ion ratios. Quantifying ion ratios for each analyte are determined by the ratio of MRM 1 (analyte)/MRM 1 (internal standard). The concentration versus quantifying ion ratio of the calibrators is used to establish a calibration curve and calculate the concentrations of the unknown samples.

(c) Retention time. The retention time for each analyte recognized in patient samples should be within 3% of the average retention times observed for the calibrators.

4. Injection volume can be adjusted depending on instrument sensitivity and the relative need for low-concentration sensitivity versus assay linearity.
5. Interferences observed include trimipramine with imipramine and cylobenzaprine with amitriptyline. Such interferences are only observed when trimipramine concentrations exceed 1,000 ng/mL. These masses are monitored for in the assay.

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Chapter 17

Quantitation of First- and Second-Generation Antipsychotics by LC-MS/MS

Elizabeth R. Kiely and Heather M. Antonides

Abstract

Antipsychotic drugs are becoming a larger part of the prescription drug market. In combination with traditional indications for prescribing these drugs, new effective therapies are proving worthwhile as well. Here, a successful method for detecting both first- and second-generation antipsychotics is presented using a solid phase extraction method and LC-MS/MS detection. This method is used for many sample matrices and can also be used for detecting antidepressants, which are often prescribed in conjunction with antipsychotics.

Key words: Antipsychotics, LC-MS/MS, Solid phase extraction, Clozapine, Risperidone, Haloperidol, Antidepressants

1. Introduction

In 2009 antipsychotics were the most commonly prescribed class of medications in the United States (1). With sales totaling $14.6 billion (1), it is surprising that this class of drugs does not receive greater attention in the toxicological analysis of either postmortem or human performance casework. With first-generation antipsychotics historically used for the treatment of schizophrenia and bipolar disorder, the second-generation, or atypical, antipsychotics have broadened the spectrum of applications for this drug class to be prescribed to treat Tourette syndrome, autism, and some withdrawal symptoms associated with opiate and alcohol addiction (1, 2). The "off label" uses range from treating anorexia to symptoms related with epilepsy (3).

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Haloperidol (Haldol), risperidone (Risperdal), loxapine (Loxitane), ziprasidone (Geodon), quetiapine (Seroquel), clozapine (Clozaril), aripiprazole (Abilify), and thioridazine (Mellaril) are targeted in this solid phase extraction (SPE), liquid chromatography—tandem mass spectrometry (LC-MS/MS) method. Both 9-hydroxyrisperidone (Paliperiodone), an equipotent metabolite, and mesoridazine (Serentil) are also included in this method as they are pharmacologically active major metabolites of risperidone and thioridazine, respectively (4). Olanzapine (Zyprexa) can be quantified with this instrument method; however, the extraction method is a liquid–liquid basic extraction (see Note 1). Due to the subsequent administration of antidepressants in conjunction with antipsychotics, this method can also be used for many of the common antidepressants, including the selective serotonin reuptake inhibitors (SSRIs) (see Note 2).

A simple SPE procedure (5, 6) is used to extract the analytes of interest from several biological matrices, including blood and liver (see Note 3). The specimens are treated with phosphate buffer and internal standard (haloperidol-d_4, aripiprazole-d_8, and quetiapine-d_8) and applied to the conditioned SPE columns. The analytes are eluted from the SPE columns using an elution solvent of methylene chloride/2-propanol/ammonium hydroxide, evaporated to dryness, and reconstituted with methanol. The samples are then injected on the LC-MS/MS using electrospray ionization and the multiple reaction monitoring mode. Specifically, the haloperidol-d_4 is used to quantify haloperidol, risperidone, 9-hydroxyrisperidone, ziprasidone, thioridazine, mesoridazine, clozapine, and loxapine. Quetiapine and aripiprazole are quantified using quetiapine-d_8 and aripiprazole-d_8, respectively. The therapeutic ranges of these drugs are encompassed in a six point curve, ranging from 10 to 1,000 ng/mL. Postmortem redistribution is addressed by analyzing liver specimens when femoral blood is not available.

2. Materials

All reagents are Certified ACS grade unless otherwise stated (see Note 4).

2.1. Extraction

1. Blank blood matrix. Bags of blood are donated by the local blood bank when they are no longer suitable for use. Each bag is pooled into a lot and analyzed under all methods that the laboratory currently utilizes (see Note 5).
2. 1 M acetic acid. Add 5.7 mL glacial acetic acid to a 100 mL volumetric flask and fill to volume with distilled water. Store at room temperature.

3. 0.1 M Acetic acid. Dilute sufficient 1 M acetic acid for the extraction, e.g., add 2.5 mL of 1 M acetic acid to 22.5 mL of water. Make fresh for each extraction.
4. 0.1 M Phosphate buffer, pH 6.0: Titrate 1 L of 0.1 M monobasic potassium phosphate to pH 6.0 with 0.1 M dibasic potassium phosphate.
   (a) 0.1 M monobasic potassium phosphate solution: Add 13.61 g of KH_2PO_4 to a 1 L volumetric flask and fill to volume with distilled water. Mix well to dissolve completely.
   (b) 0.1 M dibasic potassium phosphate solution: Add 42 g of K_2HPO_4 to a 1 L volumetric flask and fill to volume with distilled water. Mix well to dissolve completely.

   Store the monobasic solution, the dibasic solution, and the pH 6.0 phosphate buffer at room temperature.
5. 5 μg/mL Working internal standard, in methanol. Haloperidol-d_4, quetiapine-d_8, and aripiprazole-d_8 (Cerilliant, Round Rock, TX) are purchased as 100 μg/mL solutions. All are stored in the freezer until consumed. To a 10 mL volumetric flask, add 0.5 mL each stock solution. Fill to volume with methanol and mix well. Store in the freezer 0°C.
6. 10 μg/mL Working calibration standard, in methanol. Clozapine, 9-hydroxyrisperidone, haloperidol, risperidone, thioridazine, quetiapine, ziprasidone, olanzapine, aripiprazole (all from Cerilliant, Round Rock, TX), loxapine and mesoridazine (both from Grace Davison Discovery Sciences, Deerfield, IL) are purchased as 1 mg/mL solutions and stored in the freezer until consumed. To a 10 mL volumetric flask, add 100 μL each stock solution. Fill to volume with methanol and mix well. Store in the freezer 0°C.
7. Quality controls (QC) can be purchased, or a working QC standard can be prepared as described for the working calibration standard. The QC standard should be prepared from a different manufacturer and independently from the calibration standard.
8. Elution solvent. Methylene chloride/2-propanol/ammonium hydroxide (78/20/2). Ammonium hydroxide (Certified ACS Plus) and 2-propanol are combined first and then the methylene chloride (Burdick & Jackson ACS/HPLC) is added. This reagent must be made fresh during every extraction.
9. SPE columns. United Chemical Technologies, Inc. ZS/DAU 020 columns. Store in a cool dry area. If a partial bag remains, it is stored in a desiccator.

2.2. Instrument

1. Nylon solvent filters, Phenomenex filter membranes. 0.45 μm, 47 mm.
2. Mobile phase A (MP-A): 10 mM Ammonium acetate. Prepare fresh with every instrument run by adding 0.771 g of ammonium acetate to a 1 L volumetric flask and filling to volume with Burdick & Jackson ACS/HPLC grade water. Mix well to dissolve completely. Filter prior to use.
3. Mobile phase B (MP-B): 50:50 Acetonitrile:methanol. Filter prior to use. Store in the refrigerator between instrument runs.
4. Working mobile phase: 65:35 MP-B: MP-A.
5. HPLC Column. Agilent XDB-C18, 4.6×150 mm, 5 μm particle size. Store in 80/20 acetonitrile/water when not in use (see Note 6).
6. Agilent 1200 Series HPLC with 6410 Triple Quad LC/MS.

3. Methods

Postmortem redistribution is a common concern with both antipsychotic and antidepressant analytes. Another concern for toxicological analysis is second matrix confirmation. Routinely, two different matrices are used for confirming an analyte's presence. Femoral blood is considered the most accurate representation of an analyte's concentration and is most often used for defining therapeutic and toxic levels. Therefore, for this analysis an alternate blood specimen as well as a liver specimen is analyzed if femoral blood is not available.

3.1. Sample Preparation

1. 2 mL of specimen is used for the analysis of blood, urine, plasma, vitreous humor, or cerebral spinal fluid. If liver or brain is needed for the analysis, a homogenate is prepared (see Note 7). 1 mL of liver homogenate or 2 mL of brain homogenate is used.
2. Spike calibrators (and controls, if applicable) according to Table 1. Each is prepared by spiking working calibration/control standard into 2 mL of blank blood matrix. Low and high controls are analyzed in each run (see Note 8). The target concentrations of the controls should be representative of the low and high end of the calibration curve. The calibration range is 10–1,000 ng/mL (see Note 9). All controls and calibrators should be vortexed after preparation is complete.

Table 1
Calibrator and control preparation

Spike volume (µL)	Expected concentration (ng/mL)
Calibrators	
2	10
4	20
10	50
150	750
200	1,000
Controls	
5	25
160	800

3.2. Extraction

1. Pipet samples (calibrators, controls, and unknowns) as described above into 20×125 mm screw top test tubes. Add 50 µL of working internal standard and vortex thoroughly.
2. Add 8 mL of 0.1 M phosphate buffer, pH 6.0, cap and rotate specimens for 10 min. Centrifuge for 10 min at 1,000 RCF.
3. Condition the SPE columns prior to the addition of the specimens. In order, add the following one at a time to each column: 3 mL of methanol, 3 mL of water, 1 mL of 0.1 M phosphate buffer, pH 6.0. Allow each to flow by gravity.
4. Apply specimens to the columns using a transfer pipet. The addition of any sediment should be avoided as it will inhibit the specimen's ability to pass through the column.
5. After the specimen has completely passed through the column, each SPE column should be washed with the following, one at a time, in the order listed: 3 mL of water, 1 mL of 0.1 M acetic acid, 3 mL of methanol. Allow to flow by gravity.
6. The SPE columns should be dried under vacuum, >10 mmHg, for at least 30 min.
7. Elute the specimens with the methylene chloride/2-propanol/ammonium hydroxide (78/20/2) into 16×100 mm disposable glass tubes (see Note 10).
8. Evaporate to dryness with nitrogen at 40°C.
9. Reconstitute with 200 µL of methanol, vortex for 30 s, and transfer to autosampler vials.

3.3. Instrument Preparation

1. Filter the mobile phase components (50:50 acetonitrile: methanol and 10 mM ammonium acetate).
2. Pump each mobile phase component at 5 mL/min for 5 min with the instrument purge valve open to flush lines and prime instrument (7). If the instrument is not sufficiently primed, retention times may be outside their monitoring window or chromatographic peaks may be absent.
3. Check for leaks and turn on the entire system to allow for the column and mass spectrometer to equilibrate at the set parameters. See Tables 2 and 3 for instrument and ion parameters (see Note 11).
4. After mobile phases are primed and instrument is in the ready state, it is necessary to Autotune or Checktune (see Note 12).
5. After Autotune or Checktune is complete, ensure all parameters are loaded for the analysis, wait for instrument ready, and inject a test mix to ensure all retention windows are appropriately set. An example chromatogram is shown in Fig. 1.

Table 2
Antipsychotic instrument parameters

Source parameters		
Gas temp (°C)		350
Gas flow (L/min)		12
Nebulizer (psi)		55
Capillary (V)		4,000
Time segments (min)		
1	0–2	
2	2–7.2	
3	7.2–11	
4	11–17	
5	17–29	
Injection amount		5 µL
Flow (mL/min)		0.8
Run time		29 min
Solvent ratio A: 10 mM ammonium acetate B: Methanol:acetonitrile (50:50)		35A:65B
Column temp (°C)		50

Table 3
Antipsychotic ion parameters

Analyte	Precursor ion	Product ion	Dwell (ms)	Fragmentation (V)	Collision energy (V)	Ret. time (min)
Time segment 2						
9-Hydroxyrisperidone	427.2	427.2	40	148	0	3.1
	427.2	**207.1**	40	148	32	
	427.2	110	40	148	48	
Mesoridazine	**387.2**	**372.1**	40	143	20	3.3
	387.2	274	40	143	25	
Risperidone	411.2	411.2	40	133	0	3.8
	411.2	**191.1**	40	145	36	
	411.2	110	40	145	60	
Haloperidol-d_4	**380.2**	**169**	40	132	24	3.9
	380.2	127	40	132	25	
Haloperidol	376.2	376.2	40	125	0	4.0
	376.2	**165**	40	135	24	
	376.2	123	40	135	48	
Olanzapine	313.2	313.2	40	137	0	4.5
	313.2	**256.1**	40	135	24	
	313.2	198	40	135	48	
Quetiapine-d_8	393	393	40	138	0	6.1
	393	**258.1**	40	138	24	
Quetiapine	384	384	40	150	20	6.3
	384	279	40	150	20	
	384	**253**	40	150	20	
Olanzapine	**327.2**	**270.1**	40	140	24	6.4
ethyl analog	327.2	84.1	40	140	24	

(continued)

Table 3 (continued)

Analyte	Precursor ion	Product ion	Dwell (ms)	Fragmentation (V)	Collision energy (V)	Ret. time (min)
Time segment 3						
Clozapine	327.1	327.1	100	127	0	8.1
	327.1	**270**	100	135	24	
	327.1	192	100	135	52	
Ziprasidone	413.1	413.1	100	150	0	10.0
	413.1	**194**	100	140	32	
	413.1	159	100	140	52	
Time segment 4						
Thioridazine	371.2	371.2	100	125	0	11.4
	371.2	**126.1**	100	130	24	
	371.2	98	100	130	36	
Loxapine	328.1	328.1	100	132	0	13.9
	328.1	**271**	100	120	24	
	328.1	193	100	120	56	
Time segment 5						
Aripiprazole-d_8	**456.2**	**293.1**	100	155	28	24.2
	456.2	106.1	100	155	44	
Aripiprazole	448.2	448.2	100	163	0	26.2
	448.2	**285.1**	100	160	28	
	448.2	176	100	160	36	

Bold text denotes the mass transition used for quantitation

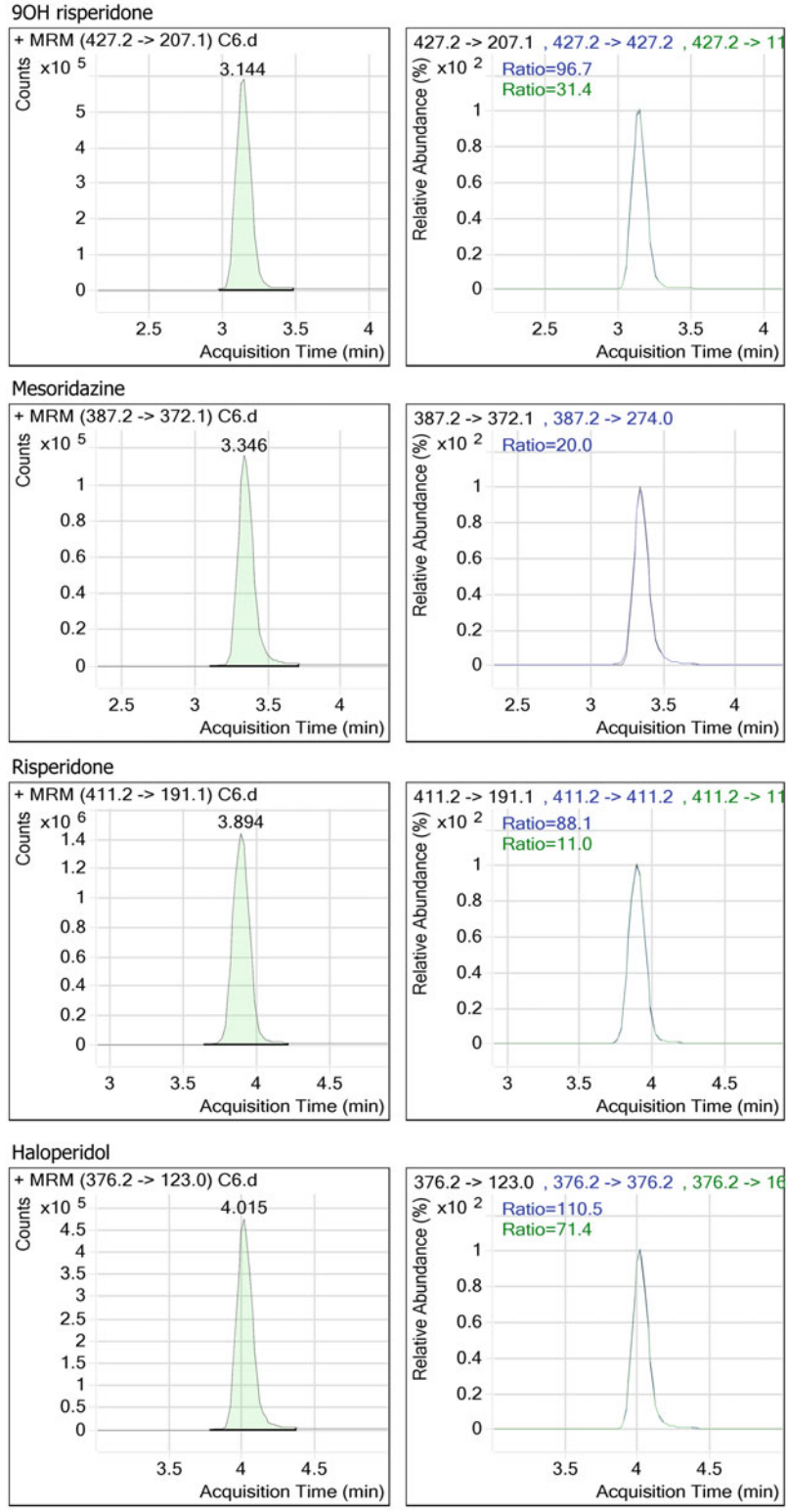

Fig. 1. Extracted ion chromatograms for antipsychotic analytes and internal standards.

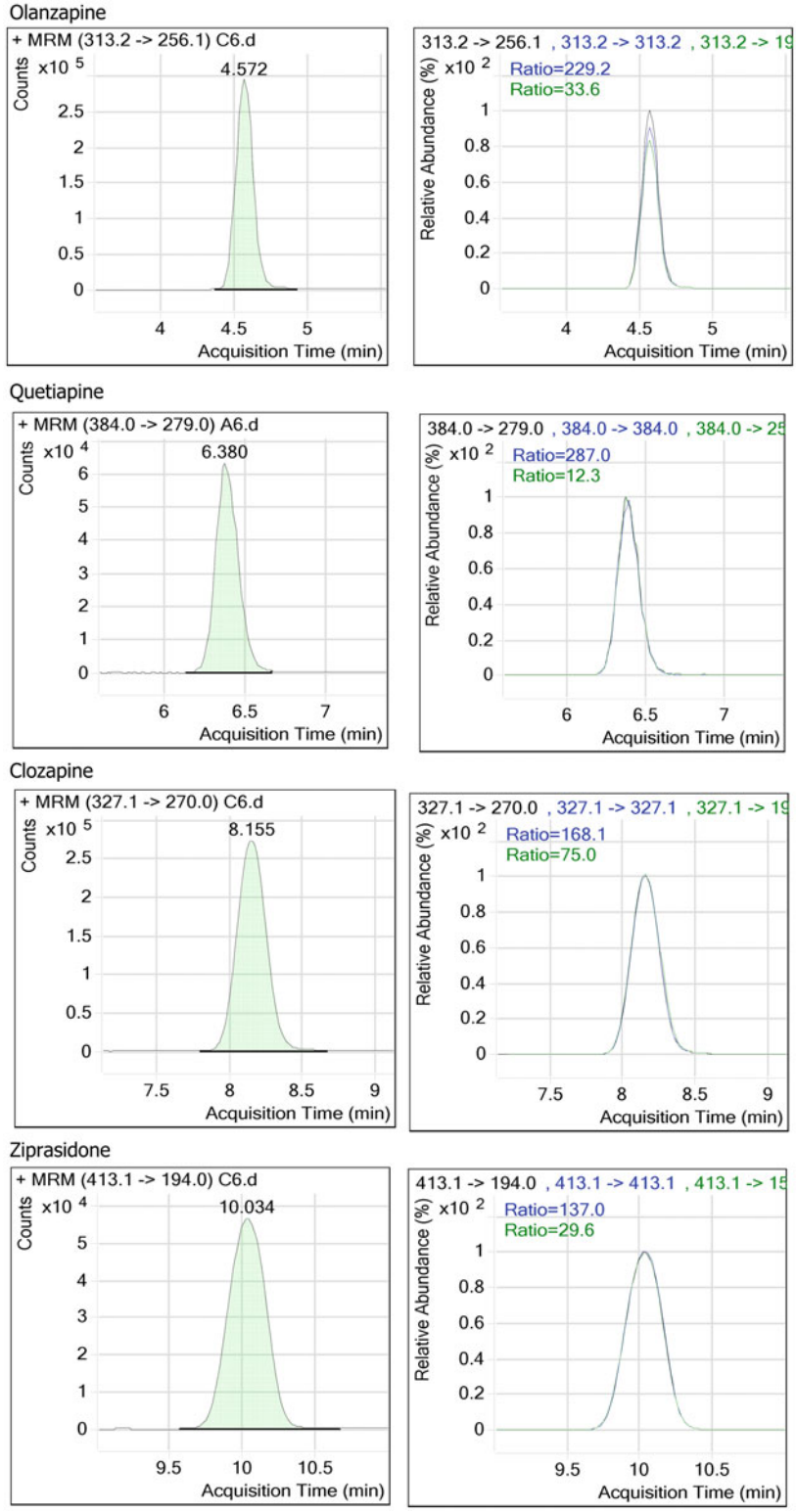

Fig. 1. (continued)

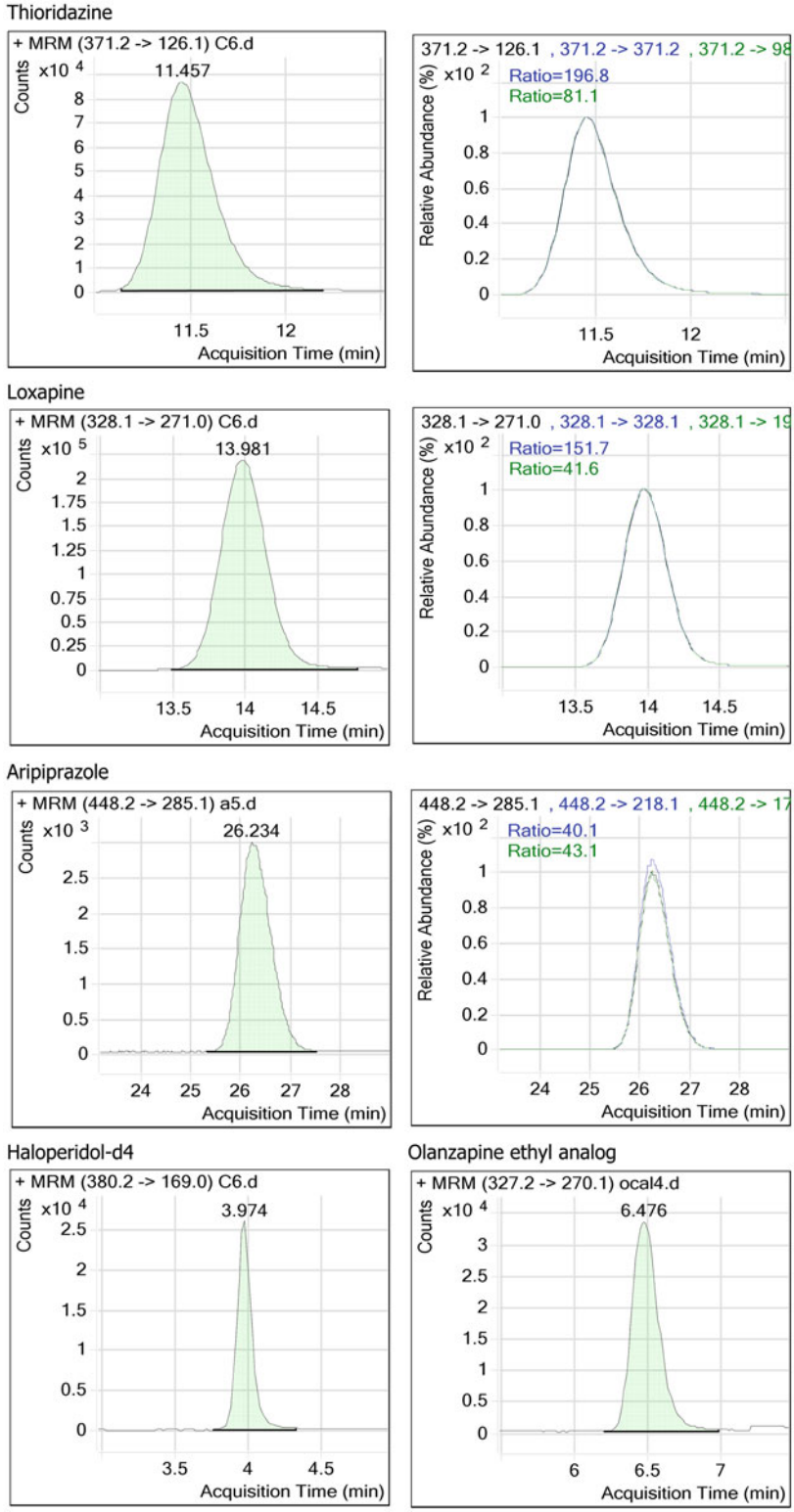

Fig. 1. (continued)

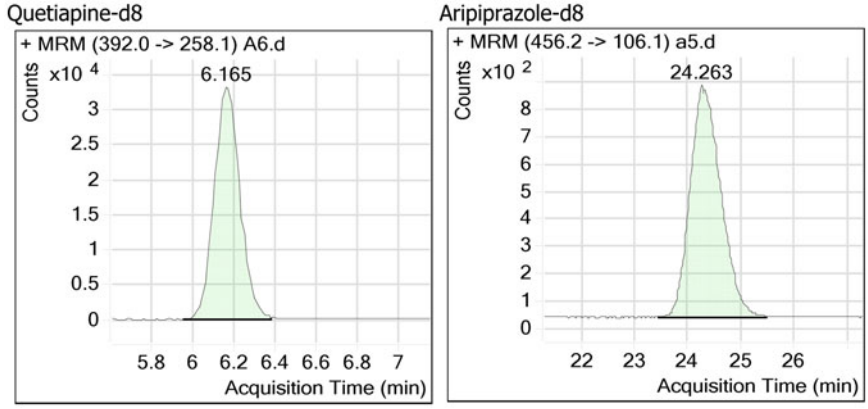

Fig. 1. (continued)

4. Notes

1. The olanzapine liquid–liquid extraction differs from the SPE extraction in that the optimal pH for olanzapine is 9. This extraction targets olanzapine alone (8). Furthermore, it is necessary to freeze samples that are suspected of containing olanzapine due to the oxidation of olanzapine to olanzapine-S-oxide in vitro. The freezing of the specimens prior to analysis and the addition of ascorbic acid during the extraction work to limit this reaction.
2. The antidepressants that also can be detected with this method are fluoxetine, norfluoxetine, paroxetine, sertraline, norsertraline, venlafaxine, norvenlafaxine, amitriptyline, nortriptyline, doxepin, nordoxepin, imipramine, desipramine, bupropion, and hydroxybupropion. Cyclobenzaprine is also detectable using this method. Paroxetine-d_6 is used as the internal standard for all analytes. The extraction method is the same for the antipsychotics, as are the instrument parameters. The calibration range for all analytes is 25–1,500 ng/mL.
3. Other matrices that have been analyzed successfully with this method are cerebral spinal fluid, vitreous humor, brain, urine, and bile. Bile is run at a 1/2 dilution in saline to facilitate the sample through the solid phase extraction column.
4. We have found that the LC-MS/MS system performs better with Burdick & Jackson reagents. The cleaner the mobile phase used, the less the instrument becomes dirty during a run, ensuring a uniform analysis from start to finish.
5. The blank blood matrix is stored in 50 mL screw top containers. Approximately 1 g of sodium fluoride is added to each

50 mL tube to prevent degradation. The containers are then stored frozen until needed.

6. After each instrument run, the column is rinsed with acetonitrile/water (80/20) at a flow rate of 0.2 mL/min for at least 1 h or five column volumes. The column is also stored in this environment until the next run to prolong column life (9). To extend the life of the columns for as long as possible, at times it is necessary to clean a column. To clean reversed phase C-18 columns, rinsing with 10 column volumes of each of the following can be done in the order listed: 95% water/5% acetonitrile (for buffer removal), tetrahydrofuran (THF), 95% acetonitrile/5% water, mobile phase of interest (9).
7. Liver and brain homogenates are prepared by using a stainless steel laboratory blender to homogenize 10 g of tissue and 30 mL of saline solution. This homogenate is then considered a 1/4 dilution. All raw results for brain homogenates are multiplied by a factor of 4 to achieve the final concentration. Due to the increased levels in the liver specimens, we only use 1 mL of homogenate (bring to 2 mL with water), thus creating a 1/8 dilution. Final concentrations are then calculated by multiplying the raw result by a factor of 8.
8. 10% of the samples analyzed are controls. This can be achieved by extracting two each of a low and high control and dispersing them throughout the instrument run to achieve this 10%. Only four controls are extracted but each is injected repeatedly throughout the run. Many control manufacturers can accommodate control needs with concentrations and analytes specific to your analysis.
9. Due to the large calibration range, the linear curve is manipulated using the 1/x function. This puts greater emphasis on the lower end of the curve, which is consistent with the therapeutic range of the analytes.
10. Occasionally, after eluting the samples with the methylene chloride/2-propanol/ammonium hydroxide mixture, some aqueous bubbles may be noticeable. Remove any aqueous by drawing off with a disposable pipet. The extract will be cleaner for injection.
11. All fragmentor voltages and collision energy settings were determined by using the Optimizer software specific to the Agilent LC-MS/MS. This software allows the user to enter the chemical formula or the molecular weight of the analyte of interest, and by varying the fragmentor voltage and collision energy through a series of injections, obtain a list of the most abundant ions in conjunction with the operating fragmentor voltage or collision energy for these ions. All product ions were

determined using this program. The mass transition used for quantitation is the most abundant product ion for that specific analyte. For the majority of the analytes, the unfragmented precursor ion and the second most abundant product ion are used for qualifier transitions. Mesoridazine is unusual in that only one transition is used for a qualifier. An interference was observed with the other product ions suggested by the Optimizer program which affected the consistency of the ion ratios. Using fragmentor voltages or collision energies that are too high can lead to extensive ionization that leaves each analyte indistinguishable from the next. The optimal setting for the instrument would be for the retention times and specific ions for each analyte to be different. When both of these cannot be achieved, the characteristic pattern of ionization for that analyte has to be relied upon. In addition, time segments are used throughout the instrument run to limit the number of ions monitored during specific times and improve sensitivity.

12. It is only necessary to Autotune the LC-MS/MS as needed, i.e., if the Checktune fails (10). During the Autotune, the parameters that are out of range are adjusted by the MSD. In order to keep the system parameters consistent, we perform an Autotune once a month regardless of the Checktune pass or fail. All other runs should be preceded by a Checktune (either positive or negative mode). During the Autotune, the instrument should be tuned in both positive and negative mode to ensure that the capillary does not become charged. The Checktune can simply be done in the mode of interest. While LC-MS/MS instruments do not have set values for the Autotune/Checktune, keeping track of certain ions over time will indicate how dirty the system is becoming. We monitor an ion that is in our range of interest (m/z 322) for both MS1 and MS2. Over time, the abundances will decrease indicating that the capillary requires cleaning.

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Chapter 18

Analysis of Selected Anticonvulsants by High Performance Liquid Chromatography-Tandem Mass Spectrometry

Jennifer A. Collins and Gregory C. Janis

Abstract

A method for the analysis of the basic antiepileptic compounds felbamate, lamotrigine, carbamazepine, carbamazepine-10,11-epoxide, gabapentin, pregabalin, levetiracetam, and oxcarbazepine monohydroxy derivative (oxcarb MHD) in human plasma is described. This protocol incorporates a simplified sample preparation step followed by quantitative high performance chromatography-tandem mass spectrometry detection of commonly prescribed and monitored anticonvulsant drugs. Since polytherapy is common in epilepsy patients, use of a multiconstituent assay can improve laboratory efficiency and reduce required analytical time.

Key words: Anticonvulsants, Seizure disorders, Therapeutic drug monitoring, High performance liquid chromatography-tandem mass spectrometry, Electrospray

1. Introduction

Therapeutic drug monitoring (TDM) of anticonvulsants is an important tool in the management of patients with epilepsy. Seizure disorders currently affect more than two million individuals in the United States with at least 140,000 new cases diagnosed annually (1). The worldwide prevalence is estimated to be 50 million (2). The frequency, intensity, and type of seizures are highly variable in this patient population. Those whose condition is controlled by medication may not experience seizures at all, whereas others continue to have seizures despite medical and/or surgical intervention. While the physiology of seizures has not been fully elucidated, animal models suggest that a reduction in inhibitory synaptic activity and/or enhancement of excitatory synaptic activity can trigger a seizure (3). Thus, individuals diagnosed with the disease are treated with a variety of anticonvulsant drugs that control

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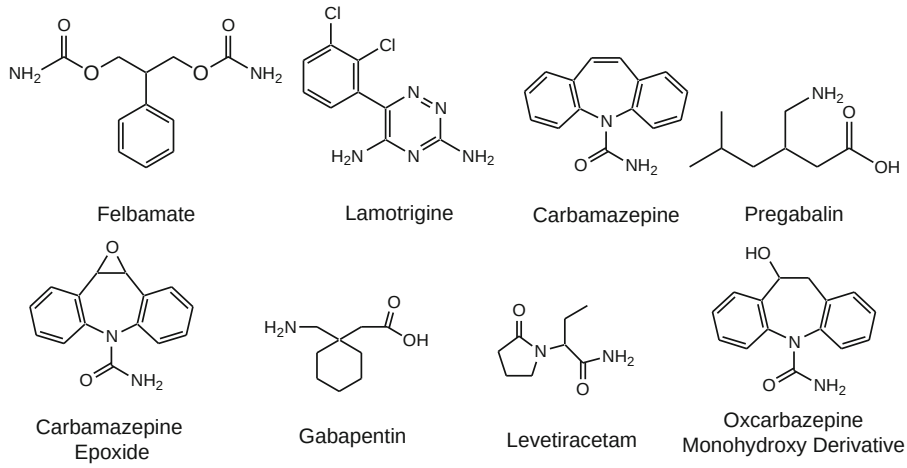

Fig. 1. Chemical structures of selected anticonvulsant compounds.

seizures by inhibiting high frequency neuronal firing patterns or enhancing GABA-mediated synaptic inhibition to raise the seizure threshold. Because of the variable nature of the disease, drug therapy must be carefully optimized for the individual patient to prevent seizures while minimizing adverse effects. This often requires routine determination of anticonvulsant blood levels and adjustment of drug dosages in order to optimize efficacy. While some patients' seizures are controlled with a single medication, polytherapy is common which requires measurement of more than one anticonvulsant drug as a part of the TDM paradigm.

There are several drugs used in the treatment of seizure disorders. Phenobarbital was the first synthetic agent recognized as having antiseizure activity (in 1912) and most of the early anticonvulsants were chemically related (phenytoin, primidone, ethosuximide). Antiseizure drugs introduced after 1965 and the newer anticonvulsants exhibit a wide diversity in chemical structures. Those drugs include carbamazepine, valproic acid, felbamate, gabapentin, lamotrigine, topiramate, tiagabine, levetiracetam, oxcarbazepine, zonisamide, pregabalin, lacosamide, rufinamide, and benzodiazepines such as clonazepam (Fig. 1). Methods utilized to measure serum/plasma concentrations of the anticonvulsants include immunoassay and a variety of chromatographic techniques.

Commercial immunoassays have long been available for the "classic" anticonvulsants such as phenytoin, carbamazepine, valproic acid, primidone, and ethosuximide and generally provide useful results under normal TDM conditions. Kits for the newer anticonvulsants such as lamotrigine, gabapentin, zonisamide, and levetiracetam have become available only recently so their effectiveness in monitoring in a broad population cannot yet be evaluated. While targeted immunoassay can be a very efficient and cost-effective way

to monitor drug levels, limitations of the methodology can impact the usefulness of this technique. Cross-reacting or interfering compounds may lead to false elevations or reductions in measured drug concentrations. In addition, failure of an immunoassay to cross-react with an active metabolite can provide misleading clinical data related to toxicity as in the case of the 10,11-epoxide metabolite of carbamazepine (4). Finally, it is important in some situations to measure only the active, free drug concentration and the sensitivity of the immunoassay may not be sufficient to obtain accurate results.

A variety of chromatographic techniques have been used for routine analysis of anticonvulsant drugs; both gas chromatography (GC) (5–8) and high performance liquid chromatography (HPLC) (9–12) methods have been published. While chromatographic methods are generally more labor intensive than immunoassay, they provide the ability to accurately quantify drug and metabolite levels in plasma and are preferred in situations where independent assessment of parent drug and metabolites or measurement of free drug levels are important. More recently, the availability of robust interfaces between liquid chromatography (LC) systems and mass spectrometers (MS) has facilitated the development of LC-MS and LC-MS/MS methods for measuring one or more anticonvulsant drugs (13–17). Although the instrument systems required for these coupled analytical techniques can be costly, the advantages of LC-MS/MS over traditional HPLC or GC methods include reduced sample volumes and sample handling requirements, shorter run times, enhanced specificity, and the ability to detect multiple anticonvulsants in a single sample. A method for analysis of eight anticonvulsant drugs by LC-MS/MS is presented.

The following method describes the analysis of the basic antiepileptic drugs felbamate, lamotrigine, carbamazepine, carbamazepine-10,11-epoxide, gabapentin, pregabalin, levetiracetam, and oxcarbazepine monohydroxy derivative (oxcarb MHD) in human plasma using a combination of zinc sulfate and acetonitrile-induced protein precipitation followed by LC-MS/MS.

The basic antiepileptic drugs felbamate, levetiracetam, and oxcarb MHD are analyzed over the concentration range of 2–100 μg/mL; carbamazepine, gabapentin, lamotrigine, and pregabalin are analyzed over the concentration range of 0.5–25 μg/mL; and carbamazepine-10,11-epoxide is analyzed over the concentration range of 0.25–12.5 μg/mL using piracetam, methylcarboxamide, and meprobamate as internal standards. Chromatographic analysis utilizes an isocratic mobile phase of 10 mM ammonium acetate with 0.1% formic acid and methanol at a flow rate of 400 μL/min through a Thermo Hypersil Prism RP column (150.0 × 2.0 mm, 5 μm). The analytes are then detected and quantified using a triple quadrupole mass spectrometer set to monitor the specific, molecular fragmentation reactions of each compound of interest.

2. Materials

2.1. Equipment

1. LC-MS/MS: Agilent 1100 HPLC-Micromass Ultima or Agilent 1200 HPLC—Applied Bioscience Sciex API 4000.
2. Thermo Hypersil Prism RP (or equivalent) HPLC column of the dimensions (150 × 2.0 mm, containing 5 μm particles).

2.2. Reagents

1. 10 mM Ammonium acetate with 0.1% formic acid. Add approximately 1,500 mL distilled water to a 2,000 mL volumetric flask. Add 1.54 g of ammonium acetate (98%, ACS grade) and 2 mL of concentrated formic acid (ACS Grade). Bring to volume with distilled water.
2. 100 mM Zinc sulfate heptahydrate. Add approximately 100 mL distilled water to a 200 mL volumetric flask. Add 5.7 g of zinc sulfate heptahydrate (99%, ACS Grade). Gently swirl until fully dissolved. Bring to volume with distilled water and mix well.
3. 50:50 Acetonitrile:water. Mix 50 mL of acetonitrile (HPLC Grade) and 50 mL of distilled water in a suitable, sealable container.
4. 50:50 Methanol:water. Mix 500 mL of methanol (HPLC Grade) and 500 mL of distilled water in a suitable, sealable container.

2.3. Reference Standards

The following standard solutions are utilized in the procedure outlined herein. All reference materials are commercially available.

1. Internal Standard Solutions (see Note 1).
   (a) Meprobamate Stock: 1.0 mg/mL. Purchased as a methanolic solution 1.0 mg/mL solution.
   (b) Piracetam Stock: 1.0 mg/mL. Quantitatively weigh 10.0 mg of piracetam into a 10 mL volumetric flask. Dilute to volume with methanol. Store at 4°C.
   (c) Methyl Carboxamide Stock: 1.0 mg/mL. Quantitatively weigh 5.0 mg of methyl carboxamide (2-methyl-5 H-dibenz[b,f]azepine-5-carboxamide) into a 5 mL volumetric flask. Dilute to volume with methanol. Store at 4°C.
   (d) Internal Standard Spiking Solution: 5.0 μg/mL piracetam, 2.0 μg/mL meprobamate, 1.0 μg/mL methyl carboxamide. Quantitatively transfer 50.0 μL of the piracetam stock, 10.0 μL of the methyl carboxamide stock, and 20.0 μL of the meprobamate stock into a 10 mL volumetric flask. Dilute to volume with acetonitrile. Store at 4°C.
2. Analytical Standard Solutions
   (a) Stock Standard 1: 1.0 mg/mL carbamazepine-10,11-epoxide. Quantitatively weigh 10.0 mg of carbamazipine-10,

11-epoxide into a 10 mL volumetric flask. Dilute to volume with 50:50 methanol:water. Store at 4°C.

(b) Stock Standard 2: 2.0 mg/mL carbamazepine, 2.0 mg/mL gabapentin, 2.0 mg/mL pregabalin, and 2.0 mg/mL lamotrigine. Quantitatively weigh 20.0 mg of carbamazepine, 20.0 mg of gabapentin, 20.0 mg of pregabalin, and 20.0 mg of lamotrigine into a 10 mL volumetric flask. Dilute to volume with 50:50 methanol:water. Store at 4°C.

(c) Working Standard A: 2 mg/mL levetiracetam, 2 mg/mL oxcarb MHD, 2 mg/mL felbamate, 0.5 mg/mL carbamazepine, 0.5 mg/mL gabapentin, 0.5 mg/mL pregabalin, 0.5 mg/mL lamotrigine, 0.25 mg/mL carbamazepine epoxide. Quantitatively weigh 20.0 mg of levetiracetam, 20.0 mg of 10,11-dihydro-10-hydroxycarbamazepine (oxcarb MHD), and 20.0 mg of felbamate into a 10 mL volumetric flask. Quantitatively transfer 2.5 mL of Stock Standard 1 and 2.5 mL of Stock Standard 2. Dilute to volume with 50:50 methanol:water. Store at 4°C.

(d) Working Standard B: 0.2 mg/mL levetiracetam, 0.2 mg/mL oxcarb MHD, 0.2 mg/mL felbamate, 0.05 mg/mL carbamazepine, 0.05 mg/mL gabapentin, 0.05 mg/mL pregabalin, 0.05 mg/mL lamotrigine, 0.025 mg/mL carbamazepine epoxide. Quantitatively transfer 1.0 mL of Working Standard A into a 10 mL volumetric flask. Dilute to volume with 50:50 methanol:water. Store at 4°C.

3. Method

3.1. Sample Preparation

1. Prepare a seven point calibration curve in a matrix of plasma or serum according to Table 1. Prepare in 1.5 mL snap-cap centrifuge tubes.
2. Pipette 50 µL of sample, standard, blank, or quality control (QC) material into appropriately labeled 16 × 100 disposable glass tubes (see Note 2).
3. Add 50 µL of internal standard spiking solution into each tube, except the Matrix Blank.
4. Add 10 µL of zinc sulfate solution to each tube.
5. Add 4.0 mL of 50:50 acetonitrile:water into each tube (see Note 3).
6. Vortex samples for a minimum of 2 min.
7. Centrifuge samples at approximately 10,000 × *g* for a minimum of 5 min.

Table 1
Standard preparation

Standard number	Felbamate, levetiracetam, oxcarb MHD (μg/mL)	Carbamazepine, gabapentin, pregabalin, lamotrigine (μg/mL)	Carbamazepine epoxide (μg/mL)	Microliters of Working Standard A	Microliters of Working Standard B	Microliters of matrix
1	2.00	0.50	0.25	–	5	500
2	4.00	1.00	0.50	–	10	500
3	8.00	2.00	1.00	–	20	500
4	20.00	5.00	2.50	5	–	500
5	40.00	10.00	5.00	10	–	500
6	68.00	17.00	8.50	17	–	500
7	100.00	25.00	12.50	25	–	500

8. Transfer a portion of the supernatant into appropriately labeled autosampler vials.
9. Inject 2–10 µL of the supernatant into the LC-MS/MS system described in the following section (see Note 4).

3.2. LC-MS/MS Conditions

1. Column: Thermo Hypersil Prism RP of the dimensions 150 × 2.0 mm with a 5 micron particle.
2. Mobile phase: The assay utilizes an isocratic mobile phase of 45% 10 mM ammonium acetate with 0.1% formic acid (mobile phase A) and 55% methanol (mobile phase B) at a flow rate of 0.4 mL/min. The flow rate may be adjusted if necessitated by the system back pressure or flow limits of the electrospray ionization source of the available equipment.
3. Sample analysis utilizes an LC-MS/MS operating in positive ionization mode. Multiple reaction monitoring (MRM) transitions are listed in Table 2 (see Notes 5 and 6).
4. Three internal standards are utilized in the analysis. Table 3 lists each analyte and the recommended internal standard. Quantitation should be performed using analyte to internal standard ratios against the known concentrations of the calibration curve.

Table 2
Multiple reaction monitoring transitions

			Micromass ultima settings		AB Sciex 4000 settings	
Name	Precursor m/z	Fragment m/z	Cone	Collision energy	DP	Collision energy
Levetiracetam	171.0	126.0	20	15	36	21
Meprobamate (I.S.)	219.0	158.0	28	8	61	13
Felbamate	239.0	117.0	24	18	56	25
Carbamazepine epoxide	253.0	180.0	20	25	30	30
Oxcarb MHD	255.0	194.0	30	20	51	31
Piracetam (I.S.)	143.0	98.0	10	15	51	21
Gabapentin	172.0	154.0	30	15	46	19
Pregabalin	160.0	97.2	20	15	36	21
Lamotrigine (see Note 5)	256.0	256.0	26	11	51	13
Carbamazepine	237.0	194.0	24	18	91	31
Methyl carboxamide (I.S.)	251.0	208.0	38	18	96	27

Table 3
Internal standard recommendations

Analyte	Internal Standard
Levetiracetam	Piracetam or Methyl carboxamide
Felbamate	Meprobamate
Carbamazepine epoxide	Methyl carboxamide
Oxcarb MHD	Methyl carboxamide
Gabapentin	Piracetam
Pregabalin	Piracetam
Lamotrigine	Piracetam or Methyl carboxamide
Carbamazepine	Methyl carboxamide

4. Notes

1. Stable isotope analogs of the analytes are becoming commercially available. These should be utilized as internal standards when available.
2. QC material can be purchased commercially, or prepared in a manner similar to the procedure described for the calibrators. If made in-house, the solutions used to spike the QC should be prepared independently of the Working Standards used to spike the calibrators.
3. The volume of the acetonitrile:water solution may be adjusted to achieve greater assay sensitivity or higher assay linearity. The ratio of sample to the acetonitrile:water diluent should not be reduced below 1:10.
4. The injection volume may be adjusted to achieve greater assay sensitivity or higher assay linearity. A larger injection volume may result in a stronger signal at the lower limit of quantitation of the assay, but chromatographic peak shape abnormalities may result and the dynamic range of the assay may be reduced.
5. The analysis of lamotrigine utilizes a parent to parent transition since a strong and reliable collision-induced fragment is not available. Collision energy is applied to reduce the background signal of the transition.
6. The exact transitions and tuning parameters should be optimized by each individual laboratory. The analysis of the higher concentration and more sensitive analytes utilizes parameters which have been intentionally detuned to allow for the simultaneous analysis of all of the compounds throughout the full

desired analytical ranges. Laboratories must investigate the tuning of their available instrumentation and adjust the tuning parameters to achieve a strong signal at the bottom of the calibration curve while maintaining a linear response throughout the upper end of the calibration.

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Chapter 19

Therapeutic Drug Monitoring of Tamoxifen Using LC-MS/MS

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Abstract

Tamoxifen is a selective estrogen receptor modulator (SERM) that is used widely in the treatment of estrogen receptor positive breast cancer (ER+). Therapeutic monitoring of tamoxifen, and its metabolites N-desmethyltamoxifen (NDTam) and 4-hydroxy-N-desmethyltamoxifen (endoxifen), may be clinically useful for guiding treatment decisions. Two significant barriers to tamoxifen efficacy are: (1) variability in conversion of tamoxifen into the potent antiestrogenic metabolite, endoxifen, and (2) poor compliance and adherence to tamoxifen therapy. Therapeutic monitoring can be used to address both of these issues. Low levels of endoxifen indicate either poor compliance or poor metabolism of tamoxifen. Low tamoxifen levels would suggest poor compliance while a low ratio of endoxifen to NDTam would be indicative of poor metabolism. Solid phase extraction of patient serum followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) detection enables rapid, accurate, detection of tamoxifen, N-desmethyltamoxifen, and endoxifen.

Key words: Tamoxifen, 4-Hydroxy-N-desmethyltamoxifen, Endoxifen, N-Desmethyltamoxifen, LC-MS/MS, Pharmacogenetics, Quantitation

1. Introduction

Tamoxifen is a selective estrogen receptor modulator that is used in the treatment of all stages of estrogen receptor positive breast cancer, as well as for the prevention of breast cancer in women who are at high risk for developing the disease. Tamoxifen is widely used as an adjuvant–after the removal of the primary tumor for the prevention of recurrence. However, response to this treatment is variable and approximately 30–50% of patients in the adjuvant population experience relapse. Variation in response to therapy may be due to tumor-specific factors, such as differences in gene expression within the tumor cells, or to patient-specific factors, such as germline genetic variability or patient behavior. Therapeutic drug monitoring

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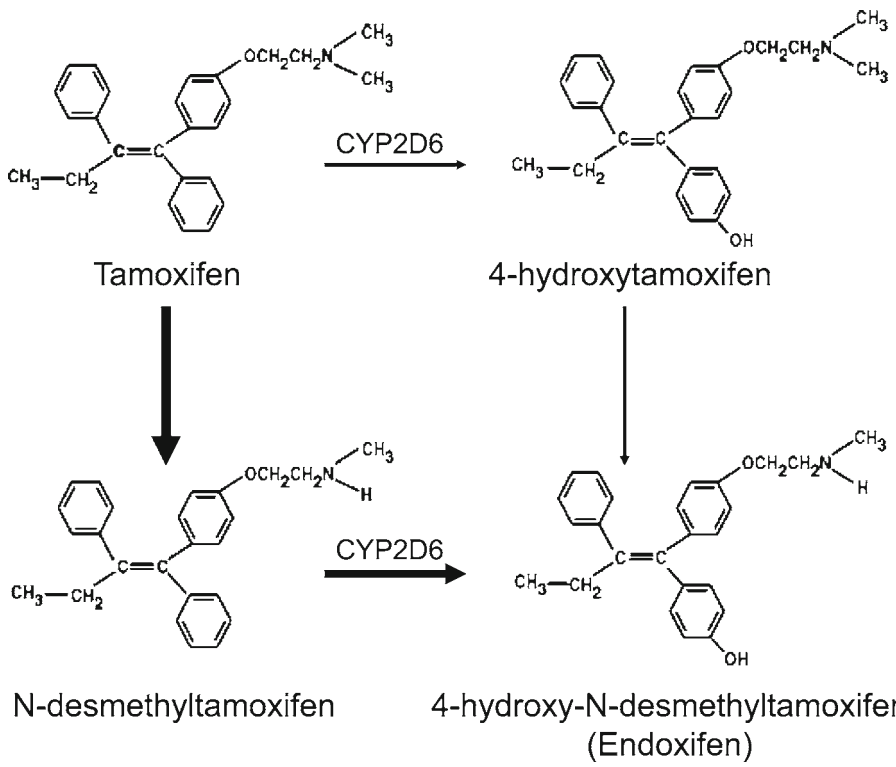

Fig. 1. The metabolic pathway of tamoxifen.

of tamoxifen and tamoxifen metabolites can be used to address some important patient-specific factors that include: (a) variation in the capacity to form potent tamoxifen metabolites due to patient genetics or concomitant medication with inhibitors of tamoxifen metabolism, or (b) poor adherence to the tamoxifen dosing regimen.

Tamoxifen is considered a pro-drug since hepatic metabolism by the CYP2D6 enzyme results in the formation of potent 4-hydroxylated metabolites [1–7] (Fig. 1). These 4-hydroxylated metabolites exhibit increased binding affinity for the estrogen receptor alpha, and are thus more potent competitive inhibitors of estrogen signaling than tamoxifen itself. Both 4-hydroxytamoxifen and 4-hydroxy-N-desmethyltamoxifen (endoxifen) are present in the serum of patients taking tamoxifen. However, because average concentrations of endoxifen are six times higher than 4-hydroxytamoxifen, endoxifen is thought to be responsible for the majority of tamoxifen's antiestrogenic effects [8]. Any factor that results in reduced serum concentrations of endoxifen could potentially compromise tamoxifen efficacy. The CYP2D6 enzyme is highly polymorphic, and a wide range of enzymatic activity exists within the human population [9]. Thus, the capacity to activate tamoxifen into endoxifen is highly variable. Polymorphisms in *CYP2D6* that result in decreased enzymatic activity have been associated with

poorer clinical outcomes in patients treated with tamoxifen in some [10–14], but not all studies [14–18]. *CYP2D6* genotype appears to predict less than 30% of the variation in endoxifen concentrations that has been observed [19]. Co-medication with potent CYP2D6 inhibitors also influences serum endoxifen concentrations. In the past, various selective serotonin reuptake inhibitors (SSRIs) were used to treat vasomotor symptoms a common side effect of tamoxifen therapy. Notably, the SSRIs paroxetine and fluoxetine are potent inhibitors of CYP2D6, and have been shown to reduce serum endoxifen concentrations in patients taking tamoxifen. These drugs are also widely used within the general population for the treatment of depression, and patients may not always inform their oncologists they are taking these drugs. Less potent inhibitors of CYP2D6 include sertraline, citalopram, celecoxib, diphenhydramine, and chlorpheniramine, but the extent to which these medications inhibit endoxifen concentrations is not well characterized. While co-medication with potent CYP2D6 inhibitors is contraindicated for patients taking tamoxifen, research suggests that drug label recommendations related to impaired bioactivation of pro-drugs are more likely to be ignored than recommendations related to adverse drug reactions [20]. When both CYP2D6 inhibitors and *CYP2D6* variants are considered, less than 50% of variation in endoxifen concentration is explained [8, 19]. Thus, there are other factors, yet to be identified, that influence serum endoxifen levels. These factors may include the use of herbal medications that interfere with tamoxifen metabolism or genetic variation that influences the elimination of endoxifen. In terms of patient behavior, compliance to adjuvant endocrine therapy is known to be poor in both premenopausal and postmenopausal patients, and this may be an additional cause of treatment failure [21–23]. For the aforementioned reasons, the direct measurement of serum endoxifen concentration may be useful in the clinical setting; low serum endoxifen concentration may predict poor response to tamoxifen therapy. Alternative, and possibly more effective, therapy can be pursued in the treatment of patients that fall into this category. In addition to using liquid chromatography mass spectrometry (LC-MS/MS) to determine if a patient is capable of conversion of tamoxifen into endoxifen, such an assay could also be used to assess compliance. By measuring tamoxifen, and the parent metabolite for endoxifen, NDTam, it may be possible to differentiate between non-compliance and poor activation. Patients who do not effectively convert tamoxifen to endoxifen may exhibit average serum endoxifen concentrations but a low NDTam:endoxifen ratio. Patients who have poor compliance will likely have low serum concentrations of all three analytes, and behavioral intervention or a change in drug therapy may be warranted.

LC-MS/MS is well suited for the quantification of tamoxifen and its metabolites in serum. Because tamoxifen and its metabolites

are small molecules, and the potent metabolites are very similar in structure to tamoxifen, measurement of concentration by immunoassay may not be possible. The use of LC rather than gas chromatography (GC) is also an advantage. Endoxifen, the metabolite of interest, is hydroxylated and would require derivatization for analysis by GC, which can be time consuming. In addition, many tamoxifen metabolites are present in the serum of patients taking tamoxifen. For this reason, MS/MS is quite useful in that specific metabolites can be detected using selected reaction monitoring (SRM). In an SRM experiment, the precursor mass of the drug and/or metabolite of interest is selected while other ions are filtered away. The precursor mass is then fragmented and a specific product ion is monitored. Drugs and metabolites with different SRM precursor:product ion pairs do not need to be completely resolved from one another for accurate quantitation, thus enabling shorter run times. The basic principles of the assay described in this chapter are: (1) resolution of tamoxifen metabolites using reverse phase chromatography, and (2) identification of metabolites using electrospray ionization (ESI) and an SRM tandem mass spectrometry method. Both retention time and identification of an SRM precursor:product ion pair are very important for quantitation since there are multiple tamoxifen metabolites that may either have the same transition or similar retention times, but not both.

One must note that the clinical utility of measuring tamoxifen and its metabolite in serum has yet to be shown. If and how this data will be used clinically is currently a subject of research. At this time, only one study has been performed looking directly at the relationship between endoxifen concentration and outcomes (WHEL, in press). The results of this study suggest that breast cancer patients undergoing tamoxifen adjuvant therapy, who have serum endoxifen concentrations in the lowest quintile, are at increased risk for breast cancer recurrence. Replication of these findings, as well as prospective trials, would increase support of the use of therapeutic drug monitoring for tamoxifen, NDTam, and endoxifen.

The major steps in the protocol for the assay described include: (1) preparation of serum or plasma samples for analysis by solid phase extraction, and (2) analysis of samples by LC-MS/MS.

2. Materials

2.1. Calibration Curve and Quality Control Material Components

1. Drug standards: tamoxifen (Sigma Aldrich), NDTam, and endoxifen (Toronto Research Chemicals). Transfer 1.0 mg of each compound into a 10 mL volumetric flask and bring to volume with methanol for a concentration of 100 μg/mL. Store at −80°C in tinted vials. (see Notes 1 and 2).

Table 1
Calibrator concentrations

Calibrator number	Concentrated drug master mix (μL)	Tamoxifen (ng/mL)	N-Desmethyl-tamoxifen (ng/mL)	Endoxifen (ng/mL)
1	160	20	40	2
2	400	50	100	5
3	1,000	125	250	12.5
4	1,600	200	400	20
5	2,000	250	500	25

2. Concentrated drug master mix: To a 10 mL volumetric flask, transfer 1.25 mL of tamoxifen, 2.5 mL of NDTam, and 125 μL of endoxifen stock standards. Bring to volume with methanol and store at −80°C in tinted vials. Final concentrations are: tamoxifen 12.5 μg/mL, NDTam 25 μg/mL, and endoxifen 1.25 μg/mL.
3. Calibration curve: To separate 100 mL volumetric flasks, add 80 mL drug-free serum. Spike with the appropriate volume of concentrated drug master mix as shown in Table 1, and fill to volume with drug-free serum. Aliquot and store at −80°C until needed.
4. QC material: High and low QC material should be prepared such that the concentrations are within the highest and lowest 1/3 of the calibration curve, respectively. For example, in separate 100 mL volumetric flasks, add 0.4 mL and 1.2 mL of concentrated drug master mix to drug-free serum, for final concentrations: tamoxifen (50 and 150 ng/mL), NDTam (75 and 300 ng/mL), and endoxifen (3 and 20 ng/mL). Aliquot and store at −80°C until needed.

2.2. Sample Preparation

1. Waters MCX 1 cc 30 mg solid phase extraction (SPE) cartridges (Waters).
2. 2% formic acid in ddH_2O. Prepare fresh.
3. 5% ammonium hydroxide (NH_4OH) in methanol. Prepare fresh.
4. D_5-tamoxifen, D_5-NDTam, and D_5-endoxifen (Toronto Research Chemicals) stocks: To separate 10 mL volumetric flasks containing approximately 8 mL of methanol, add 1 mg of each internal standard powder and dissolve. Bring to volume with methanol and store in tinted vials at −80°C. Concentration of each internal standard stock is 100 μg/mL.
5. Internal standard master mix (80×): To a 10 mL volumetric flask, add 100 μL of D_5-tamoxifen, 400 μL of D_5-NDTam, and

20 μL of D_5-endoxifen. Bring to volume with methanol. Store in tinted vials at –80°C. Final concentrations are: D_5-tamoxifen (1 μg/mL), D_5-NDTam (4 μg/mL), and D_5-endoxifen (200 ng/mL).

6. 0.5 mM ammonium formate buffer, pH 3.0: Dissolve 31.5 mg of ammonium formate in 1 L of water. Adjust pH to 3.0 using formic acid.

2.3. Liquid Chromatography

1. 50 mM ammonium formate buffer, pH 4.5 (10× master stock): Dissolve 3.15 g of ammonium formate in 1 L of water. Adjust pH to 4.5 using formic acid. Filter this stock.
2. Mobile Phase A (MP-A) 5 mM ammonium formate buffer, pH 4.5 + 2% methanol. Prepare this stock using the 10× buffer listed above in LC-MS grade water. Use LC-MS grade methanol. Check pH of this reagent before use: decant a small amount into a beaker, and then discard after testing pH. Do not insert the pH meter into the main buffer container as this may result in contamination of the mobile phase with salts that can interfere with MS detection.
3. Mobile Phase B (MP-B) 70:20:10 acetonitrile:methanol:50 mM ammonium formate buffer, pH 4.5 (by volume). Due to the nature of acetonitrile and water, the total volume of the buffer will be slightly less than the expected volumes of these reagents added together. This effect is consistent and is not a cause for concern.
4. Waters X-terra MS C18 column, 3.5 μm (2.1 mm × 150 mm), Waters 2.1 × 10 mm C18 column guard column, Pre-column (MacMod).

3. Methods

In addition to patient samples, a calibration curve and QC material should be assayed with every run. Tracking the results of QC analysis is important for determining whether or not the results from patient samples are accurate (see Note 3). Typically, multiple (>3) high- and low-concentration aliquots of QC material are prepared and run in parallel with patient samples. In order for an LC-MS/MS assay to be quantitative, there must be at least five different concentrations, in addition to a blank, in a standard curve spanning a range of physiologically relevant concentrations. The average serum concentrations for the analytes of interest are as follows: tamoxifen (100 ng/mL), NDTam (200 ng/mL), and endoxifen (12 ng/mL). However, the relative standard deviation in serum concentrations is high for all of these analytes within the patient population. This variation can range from 40 to 65%. In addition,

it must be noted that liquid stocks for NDTam and endoxifen are not commercially available at this time. Thus, it is important to standardize measurement results between laboratories.

3.1. Sample Preparation

1. A 200 µL aliquot of serum is used for analysis (see Note 4). Depending on the sensitivity of your instrumentation, more or less sample may be necessary. Calibration and QC material are run in parallel with the test samples for every run. These should be treated in exactly the same way as test samples. This assay can also be used for plasma samples (see Note 5).
2. Internal standard master-mix is spiked into 0.5 mM ammonium formate buffer, pH 3.0, and vortexed thoroughly to make sufficient working stock for the run. The master mix is 80×; for example, add 125 µL of master mix to 10 mL of buffer.
3. Add 800 µL of the internal standard working stock is added to each calibrator, control, and sample. Vortex, then centrifuge for 5 min at 16,000 × *g*.
4. Samples are extracted using Waters 1 cc MCX 30 mg SPE cartridges according to the manufacturer's protocol. A flow rate of 1 mL/min is acceptable for the load and elutes steps and a flow rate of 5 mL/min is acceptable for the washes.
   (a) Apply 1 mL of methanol to the SPE cartridge and draw through.
   (b) Apply 1 mL of ddH_2O to the SPE cartridge and draw through.
   (c) Apply sample to the SPE cartridge and draw through.
   (d) Apply 1 mL of 2% formic acid in ddH_2O to the cartridge and draw through.
   (e) Apply 1 mL of methanol to the cartridge and draw through.
   (f) Discard liquid waste and place sample tubes for elution.
   (g) Apply 1 mL of 5% NH_4OH in methanol to the cartridge and collect the eluate.
   (h) Dry samples down under a gentle stream of nitrogen at 40°C.
   (i) Resuspend in 100 µL of 80:20 MPA:MPB and transfer to autosampler vials.

3.2. Sample Analysis

1. AB Sciex Analyst Software is used to control analytical equipment during sample analysis. The instrument is allowed to equilibrate for at least 15 min prior to sample analysis. An MP-A injection is always performed prior to injections of samples, standard curve, or QC material. Injections of QC material at the beginning and end of the run (same samples injected twice) are advisable in order to ensure minimal change in detection during a run.

Table 2
Chromatography gradient

Time (min)	Flow rate (mL/min)	%MPA	%MPB
0	350	65	35
0.5	350	65	35
0.6	350	50	50
4	350	40	60
4.1	350	30	70
6	350	25	75
7	350	25	75
7.1	350	0	100
8	600	0	100
9	800	0	100
9.1	500	65	35
11	350	65	35

2. A 1200 series Agilent HPLC system is used for liquid chromatography. Column temperature is maintained at 40°C during the run. The gradient is listed in Table 2 (see Note 6).
3. An AB Sciex 3200 QTRAP® Tandem Mass Spectrometer is used for analyte detection. The SRM transitions used to monitor the analyte of interest and their internal standards are as follows: Tamoxifen: 372/72; NDTam: 358/58; endoxifen: 374/58; D_5-tamoxifen: 377/72; D_5-NDTam: 363/58; D_5-endoxifen: 379/58 (see Note 7).
4. The following conditions are used for positive mode electrospray ionization: curtain gas, 35.0; ion spray voltage, 5,500; ion source gas 1, 60.0; ion source gas 2, 35.0; and temperature, 700°C. For compound specific parameters determined using our instrumentation, see Table 3. These parameters may vary from instrument to instrument. At the end of each run, the column is cleaned with a 15 min wash with water at 300 µL/min, and then with a 100% acetonitrile wash for 15 min. The column is stored in acetonitrile when it is not in use.
5. AB Sciex Analyst Software is used for quantitation. The ratio of the peak area of each analyte in the calibration material to that of the peak area of the respective internal standard is used in order to generate a standard curve across concentrations of the

Table 3
Compound-dependent parameters

Analyte	Q1	Q3	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Cell exit potential (V)
Tamoxifen	372.2	72.1	51	8	45	4
N-Desmethyltamoxifen	358.5	57.9	41	4.5	41	4
Endoxifen	374.2	58.0	46	4.5	43	6
D_5-Tamoxifen	377.3	72.1	56	4	79	4
D_5-N-Desmethyltamoxifen	363.2	58.1	71	2.5	55	4
D_5-Endoxifen	379.3	58.0	41	8	41	4

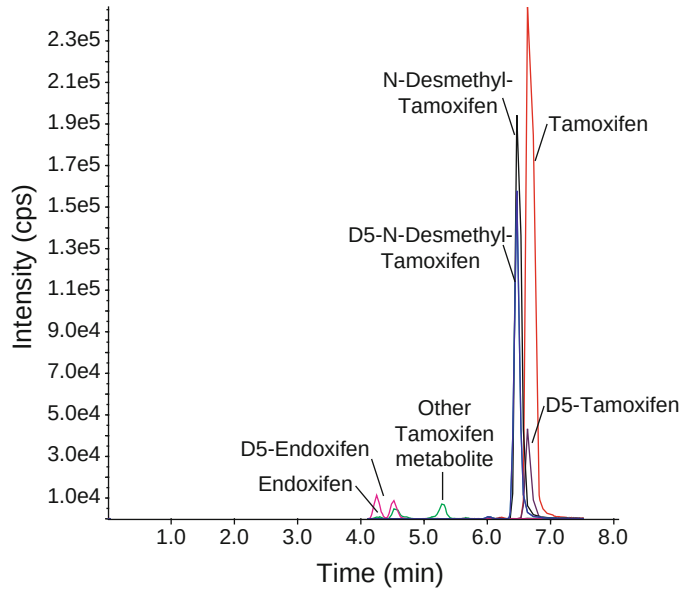

Fig. 2. An extracted ion chromatogram of a serum sample from a patient on tamoxifen.

analytes of interest. Analyst software integrates both the analyte peak area and the internal standard peak area for all analytes in unknown samples. The ratio of these peak areas is calculated. Analyst software then references the standard curve in order to determine analyte concentrations in the unknown samples. In order for the assay to be quantitative, it is imperative to include the same amount of internal standard in all samples. This allows for normalization of differences in extraction between samples, as well as possible matrix effects. An example extracted ion chromatogram is shown in Fig. 2.

4. Notes

1. Currently, the major commercial source for endoxifen and deuterated endoxifen is Toronto Research Chemicals. Endoxifen and deuterated endoxifen are called by their chemical names N-desmethyl-4-hydroxy tamoxifen and N-desmethyl-4-hydroxy tamoxifen-d_5. There may be other sources of reference compounds, but it is important to note that the quality and purity of standards may differ between suppliers.
2. There is conflicting evidence regarding the stability of tamoxifen metabolites. NDTam may break down in light, and thus samples should be stored in a dark place. Endoxifen is known to undergo spontaneous trans-isomerization [24]. However, based upon the authors' observation, the majority of endoxifen found in serum is in the *trans-* form and minimal isomerization to the *cis-* form occurs under the assay conditions described, even after several days of storage in a 4°C autosampler. Due to the presence of *cis-* and *trans-* isomers, there are two peaks present for the analyte endoxifen. This is the case for many racemic analytes. The double peak is integrated for quantitation of this metabolite. It is thus important to have quantitation software that can do this in a robust and reproducible manner.
3. While LC-MS/MS is a robust technology, there are several factors that can affect the results of the assay. For this reason, it is advisable to have checks in place to assess the status of your equipment and reagents. Pay close attention to the retention times and peak areas of analytes at known concentrations (for instance data associated with QC material). A difference of more than 10 s in retention time is notable and suggests a problem that can be attributed to the analytical column or the mobile phase. A dramatic decrease in peak area of a given analyte suggests the mass spectrometer needs maintenance. Retention time and changes in detection sensitivity can have dramatic effects on the accuracy and precision of analyte quantitation.
4. Care should be taken in the collection of samples for analysis. For instance, hemolyzed serum is technically a different matrix from normal serum and may result in additional matrix effects. Samples extracted from hemolyzed serum are rust colored and are likely to be much dirtier than properly collected serum. Also, patient health may influence assay results. Samples extracted from serum containing high levels of bilirubin appear to be tinted yellow. While these samples are relatively rare, this should be noted since quantitation of metabolites may be affected.
5. Anticoagulants do affect the sensitivity of tamoxifen metabolites by MS/MS due to matrix effects. Internal standards appear to normalize for the effects of anticoagulants. However, if plasma is to be assayed, rather than serum, the calibration curve and

QC material should be prepared in drug-free plasma containing the same anticoagulant as the samples to be tested. In addition, it is advisable to assess the sensitivity and limit of detection with different matrices using your own LC-MS/MS system. This may differ between matrices and have consequences for the precision and accuracy of detection of the assay.

6. The LC method described was designed in order to separate analytes from phospholipids and lysophospholipids, which can interfere with detection of tamoxifen and its metabolites [25]. However, certain patients have very high levels of lipids in their serum, visible to the naked eye. While SPE gets rid of a lot of interfering substances, high levels of lipids may overwhelm the capacity of these cartridges, thus resulting in samples with higher than normal levels of lipids and lysophospholipids. Again, these samples are encountered infrequently, but it may be worthwhile to note when a sample is highly lipemic.
7. There is also another tamoxifen metabolite that shares the 374/58 transition with endoxifen, the identity of which has yet to be determined. This peak is only present in the serum of patients who are taking tamoxifen. In the current method, this metabolite elutes shortly after the endoxifen peaks (Fig. 2), and it is therefore very important to resolve this metabolite from endoxifen, and check chromatograms in order to ensure the area of this peak is not integrated with endoxifen during quantitation. In an ideal system, one would be able to have both cis- and trans- endoxifen elute as a single peak, and simultaneously resolve endoxifen from the other metabolites; however, this is likely to be challenging using C18 column chemistry. Tamoxifen and tamoxifen-N-oxide (388/72) interconvert. Heme is responsible for the conversion of tamoxifen to tamoxifen-N-oxide [26]; however, it appears to be a fairly minor metabolite in serum and it is not a potent antiestrogenic tamoxifen metabolite. The significance of this interconversion, in terms of patient care, is unknown.

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