Characterization of an Amphetamine Interference from Gabapentin in an LC–HRMS Method

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Abstract

An amphetamine interference was observed during the development of an liquid chromatography–high-resolution mass spectrometry (LC–HRMS) multi-class confirmation method for the determination of 47 drugs and metabolites in urine. The interference passed all qualitative criteria for amphetamine leading to potential false-positive results. Upon investigation, it was found that the amphetamine interference was correlated with the presence of high levels of gabapentin. Gabapentin is routinely detected in patient urine specimens at levels in excess of 1 mg/mL as it is widely prescribed at high doses and does not undergo significant metabolism. The source of the interference was identified as a gabapentin in-source fragment isomeric with protonated amphetamine. Here we describe the characterization of this interference and how its effect was mitigated in the LC–HRMS method.

Introduction

Pain management and addiction clinics routinely use urine drug testing (UDT) to monitor patient compliance with treatment programs. In recent years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) UDT methods monitoring large numbers of drugs and metabolites from multiple drug classes have become more prevalent due to advantages for both the laboratory and the ordering physician (1–3). Such methods improve laboratory efficiency in an era of decreasing reimbursements and, by including a large number of relevant prescription and illicit drugs, provide clinicians with comprehensive results without extending turn-around time.

The development of multi-class assays is challenging due to the diversity of analyte structures and chemistry typically encountered and the disparity in dose regimens and relevant cutoffs/lower limits of quantitation (LLOQ). Despite the large number of analytes, the method should have a reasonable cycle time to facilitate throughput and be free from interferences that could produce erroneous results. Interferences may arise from multiple sources, including endogenous compounds and other drugs or metabolites not monitored as analytes. During the development of a liquid chromatography–high-resolution mass spectrometry (LC–HRMS) multi-class confirmation method for 47 analytes, we encountered numerous potential interferences. A description of opioid interferences has been published (4). Additionally, we observed an amphetamine interference that produced potential false-positive results for several patients. Upon investigation, it was found that these results were correlated with the presence of high levels of gabapentin.

Gabapentin was approved by the Food and Drug Administration (FDA) in 1993 for adjunctive treatment of partial seizures and marketed as the brand Neurontin (Pfizer). Subsequent indications include 2002 approval for the treatment of post-herpetic neuralgia. Since 2004, gabapentin extended release formulations have been approved (Gralise®, Horizant®) and, along with generics, have contributed to its September 2017 ranking as the seventh most-prescribed medication in the USA (5). According to a report by Information Medical Statistics (IMS) Health, 68 million prescriptions for gabapentin were written in the USA in 2017, a 54% increase since 2013 (6).
proliferation in gabapentin prescriptions is a reflection of aggressive marketing strategies for the promotion of its off-label use and clinicians looking for safer alternatives to opioids for the management of chronic pain and substance use disorders (5, 7, 8). Alone or in combination with other drugs, it has been estimated that 95% of gabapentin prescriptions are for off-label indications, including the treatment of fibromyalgia and other chronic pain syndromes, psychiatric disorders, migraine, insomnia, multiple sclerosis and opioid, cannabis, benzodiazepine and alcohol addiction (9–13).

Gabapentin is typically prescribed as three divided doses for a total of 900–1800 mg/day but can be increased up to 3600 mg/day. In human, gabapentin is not appreciably metabolized and is eliminated largely unchanged by renal excretion (14). These factors, combined with the surge of gabapentin prescriptions, explain the relatively common occurrence of gabapentin concentrations in excess of 1 mg/mL in patient urine specimens (in-house results).

The challenges of accommodating very high gabapentin concentrations in UDT have been reported by Shugarts (15). In this work, amphetamine and its internal standard displayed poor peak shape, drastically reduced peak areas and retention time shifts due to gabapentin column overload. Affected samples were reflexed to an alternative confirmation method until both the sample preparation and LC–MS/MS methods were re-developed. We observed similar chromatographic issues from high gabapentin levels, but additionally we observed an interference peak having the same m/z as amphetamine (m/z 136.1121). The interference passed all qualitative criteria for amphetamine leading to potential false-positive results. Here we describe the characterization of this interference and how its effect was mitigated in the LC–HRMS method.

**Experimental**

**Chemicals and reagents**

Gabapentin Related Compound A was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Deuterated water, deuterated formic acid and gabapentin solid used for hydrogen–deuterium exchange experiments were purchased from Sigma–Aldrich (St. Louis, MO). All method analytes and their respective internal standards, including gabapentin-D10, were purchased from Cerilliant (Round Rock, TX). Surine™ DOA synthetic urine, used for the preparation of calibrators and quality control (QC) samples, was purchased from Dyna-Tek Industries (Lenexa, KS). For mobile phase preparation, concentrated formic acid (98%, ACS grade) was purchased from EMD Millipore (Billerica, MA) and methanol (LC/MS grade) was purchased from Thermo Fisher Scientific (Waltham, MA). Purified water was supplied by an in-house MilliQ® system (EMD Millipore). IMCSzyme® recombinant β-glucuronidase and Rapid Hydrolysis Buffer (pH 7.2–7.8), used for enzymatic hydrolysis, were purchased from IMCS (Integrated Micro-Chromatography Systems, Irmo, SC).

**Sample preparation**

Multi-analyte reference stock solutions were prepared in methanol and stored at ~20°C. Working calibrators and QCs were prepared in Surine™ (synthetic urine). The patient specimens used for method development were a subset of those routinely submitted to Dominion Diagnostics by pain management and addiction clinics for compliance monitoring. The specimens were collected as random urines and represented a wide range of parent drug and metabolite levels. Calibrators, QCs and patient specimens were prepared for analysis in 96-well plate format using Freedom EVO® liquid handling systems (Tecan US, Morrisville, NC). Samples and specimens (75 μL) were diluted with 300 μL of a composite mixture containing internal standards, β-glucuronidase and Rapid Hydrolysis Buffer. Plates were sealed, gently vortexed for 1 min, incubated for 1 h at 65°C and centrifuged at 2,272 g for 7 min.

**LC–MS/MS analysis**

The primary LC–MS/MS system used for method development studies comprised an Acquity UPLC® I-Class (Waters Corp., Milford, MA) and Q ExactiveTM hybrid quadrupole-Orbitrap™ high-resolution accurate mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A Poroshell™ 120 SB-C18 column (2.1 × 50mm, 2.7-μm particle size) was used for the gradient chromatographic separation (Agilent Technologies, Santa Clara, CA) with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in methanol as mobile phase B. The flow rate
was 0.5 mL/min, and gradient conditions were as follows: 5% B (0.00–0.50 min), 5–25% B (0.50–1.50 min), 25–60% B (1.50–4.60 min), 60–95% B (4.60–4.61 min), 95% B (4.61–5.00 min), followed by re-equilibration at initial conditions (total cycle time 5.5 min). The Q Exactive source conditions were as follows: sheath gas flow rate, 65; auxiliary gas flow rate, 20; spray voltage, 3.50 kV; capillary temperature, 300°C; S-lens RF level, 55; and auxiliary gas heater temperature, 420°C. TraceFinder™ 3.2 was used for data acquisition and processing. Thermo Xcalibur™ 3.0 was also used for data processing.

The Q Exactive acquisition method comprised a full scan (m/z 130–480 at 70,000 resolution) followed by MS/MS scans (17,500 resolution with a 0.9 m/z isolation window) triggered from a Data-Independent Acquisition inclusion list. Analyte identity was established relative to a standard by scoring the following qualitative criteria using TraceFinder 3.2: retention time (RT ± 0.15 min), full-scan accurate mass (±5 ppm window), full-scan isotope pattern (score range: 0–100; a score ≥ 70 was used as the positive cutoff), MS/MS accurate mass for at least two expected fragments (±10 ppm window) and MS/MS spectrum. A custom MS/MS library was built in-house using MS/MS spectra derived from standards. Thermo Library Manager™ 2.0 and NIST08 Mass Spectral Library (user library feature) were used within TraceFinder 3.2 to search the custom library and score the qualitative criteria. A threshold score was established to confirm identity (score range: 0–100; a score ≥60 was used as the positive cutoff). No weighting was applied to the scoring criteria. If an analyte passed all the qualitative criteria stated above, it was considered positive. Analyte quantitation was performed using the peak area ratio from the full-scan extracted ion chromatograms (XICs) of the analyte and its internal standard; the XIC mass window was the accurate mass (±5 ppm) in all cases.

Results and Discussion

An amphetamine interference (m/z 136.1121) was observed in patient samples during the development of an LC–HRMS confirmation method for 47 analytes. The interference passed all qualitative criteria for amphetamine leading to potential false-positive results. Upon review of the amphetamine interference in affected patient samples, a distorted peak was noted with shifted retention time. The expected amphetamine retention time was 1.58 min; the interference peak eluted slightly earlier. A shift and suppression of the amphetamine-D5 internal standard peak was also observed (Figures 1A–D).

It was noted that the affected patient samples were all positive for gabapentin, 1 of the 47 analytes monitored. The amphetamine interference peak was observed at the gabapentin retention time and mirrored the poor gabapentin peak shape observed in the affected patient samples attributed to high gabapentin levels and column overload. The expected gabapentin retention time was 1.62 min and was generally shifted earlier under overload conditions; a corresponding shift and suppression of the gabapentin-D10 internal standard peak was also observed (Figures 1E–H). Additionally, the magnitude of the amphetamine interference was found to be highly correlated with gabapentin concentration.

To understand the apparent relationship between the amphetamine interference and gabapentin, Xcalibur was used to propose molecular formulas for the in-source fragments observed in the gabapentin MS spectrum using a ±5 ppm tolerance. Protonated gabapentin (C$_{9}$H$_{18}$O$_{2}$N) is detected at m/z 172.1332. The fragment at m/z 154.1226 (C$_{9}$H$_{16}$ON, 5% relative abundance) corresponds to the loss of one water molecule; the fragment at m/z 136.1121 (C$_{9}$H$_{14}$N, 0.002% relative abundance) corresponds to the loss of two water molecules (Figures 2A and B). The m/z 136.1121 fragment is isomeric with protonated amphetamine and was assigned as the source of the amphetamine interference.

The structure of m/z 154.1226 has been proposed to be a lactam, generated from the reaction of the gabapentin amine and carboxylic acid groups. This hypothesis was supported by the detection of an LC–MS/MS fragment ion at m/z 134.0994, corresponding to the loss of water from the lactam structure (C$_{9}$H$_{15}$O$_{2}$). The results of the LC–HRMS analysis were confirmed by the detection of the gabapentin lactam in blank samples spiked with gabapentin and the gabapentin lactam standards.

Figure 3. Structures of gabapentin (A) and gabapentin lactam (B).
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Figure 4. MS/MS spectra of gabapentin m/z 154.1226 in-source fragment from a patient urine specimen positive for gabapentin (A) and m/z 154.1226 from a gabapentin lactam standard (B).

Figure 5. MS/MS spectra of amphetamine from a standard (A) and amphetamine interference from a patient urine specimen positive for gabapentin (B).

Gabapentin lactam (Figure 3), also known as Gabapentin Related Compound A, is a gabapentin degradant and itself a pharmaceutical agent with its own range of properties (17, 18). The presence of gabapentin lactam in gabapentin drug products is known and monitored due to established toxicity (19, 20). As such, it is commercially available and was purchased to investigate its chromatographic and spectral behavior relative to the observed interference.

Under the method conditions, gabapentin lactam (retention time 3.54 min) was well separated from gabapentin (retention time 1.62 min) and was detected in both patient samples and calibration standards with peak areas <1% of the corresponding gabapentin peak areas. The levels detected are assumed to be derived from gabapentin drug products and the reference standard from which the standards were prepared. An unidentified impurity/degradant peak is apparent in the reference standard certificate of analysis (Cerilliant Catalog No. G-007) and is presumed to be gabapentin lactam. Gabapentin lactamization is known to increase with temperature and both high- and low-pH conditions (21). A comparison of gabapentin lactam levels from incubation at method conditions (65°C, neutral pH, 1 h) and at room temperature showed no increase in gabapentin lactam from the elevated temperature. At neutral pH, the zwitterionic form of gabapentin is the predominant species in solution; this species has been shown to have the least favorable lactamization kinetics (22).

Unlike gabapentin, gabapentin lactam does not produce a m/z 136.1121 (water loss) fragment; the in-source fragment was not observed nor was the (MS/MS) product ion under a range of collision energies. The MS/MS spectra from the gabapentin m/z 154.1226 (water loss) fragment and the lactam are shown in Figure 4A and B, respectively. The m/z 136.1121 fragment is absent from the lactam spectrum and the relative intensities of the ions present are different from those in the gabapentin water loss spectrum. The gabapentin m/z 154.1226 in-source fragment has been assigned as the lactam (16); however, these differences suggest contributions to the gabapentin MS and MS/MS spectra from non-lactam forms of m/z 154.1226.

The gabapentin m/z 136.1121 in-source fragment responsible for the amphetamine interference is a minor fragment but significant due to the high gabapentin levels routinely encountered in patient urine specimens. Additional experiments were performed to characterize the amphetamine interference relative to gabapentin levels. In the 47-analyte method, gabapentin and amphetamine standards span the ranges of 20.0–1000 and 100–5000 ng/mL, respectively. To mimic specimens from patients on high-dose gabapentin regimens, higher level gabapentin standards were prepared by spiking Surine™ (synthetic urine) from 0.005–1.0 mg/mL. The gabapentin-to-amphetamine interference peak area ratio in patient samples was consistent across a range of concentrations (0.004–5.4 mg/mL) and was within ±20% of the mean gabapentin-to-
amphetamine interference ratio calculated from the gabapentin standards. It was observed that a 0.025 mg/mL gabapentin standard was the lowest concentration that produced a positive amphetamine result, just above the 100 ng/mL cutoff/LOQ, passing all qualitative criteria. Gabapentin concentrations ≥0.025 mg/mL are routinely found in urines from patients on high-dose gabapentin regimens.

To mitigate the in-source fragmentation responsible for the amphetamine interference, adjustments to source and interface voltages are generally recommended. The Q Exactive voltages are not adjustable in this regard, so an alternative means of addressing the interference was required. To ensure successful differentiation of amphetamine from the interference, the MS/MS spectrum from the interference was introduced to the library. The MS/MS spectrum of the interference is qualitatively similar to amphetamine but different in the relative intensities of the parent mass (m/z 136.1121) and main amphetamine fragments (m/z 119.0855 and 91.0542) (Figure 5A and B). By adding the interference spectrum to the library as an interference, the library search algorithm was able to successfully differentiate between the MS/MS spectrum from amphetamine and that from the amphetamine interference. In this way, if a chromatographic peak has similar retention time, accurate mass and isotope pattern, then the MS/MS library criteria fails and thereby prevents a false-positive result.

A better approach is to use a chromatographic column with both higher loading capacity and improved selectivity to maintain retention and peak shape under high-load conditions and improve the separation of interfering compounds from analytes. Core-shell columns have less capacity than traditional columns packed with fully porous particles but offer the advantages of improved peak shape, lower back pressure and faster run time. As a compromise, we found that a 4.6 × 50 mm Kinetex Phenyl-Hexyl column with 10 mM ammonium formate as mobile phase A and 0.1% formic acid in methanol as mobile phase B provided good separation of amphetamine and gabapentin, presumably taking advantage of the difference in aromatic character. The conditions also offered improved retention and peak shape for gabapentin at high levels. The Kinetex Biphenyl column with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in methanol as mobile phase B also separates amphetamine from gabapentin, but gabapentin peak shape was poor at high levels. In Shugart’s (15) re-developed method, a Kinetix F5 (pentafluorophenyl) column was used in combination with 10 mM ammonium formate as mobile phase A and 0.1% formic acid in water as mobile phase B. The evaluation of columns and conditions is on-going in our laboratory.

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