Analysis of Mitragynine and Metabolites in Human Urine for Detecting the Use of the Psychoactive Plant Kratom

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The leaves of the South Asian plant kratom are described as having stimulating effects at low doses, and opiate-like analgesic and euphoric effects at high doses. A long history of use and abuse has led to the classification of kratom as a controlled substance in its native Thailand and other South Asian countries. However, kratom is not controlled in the United States, and the ready availability of kratom has led to its emergence as an herbal drug of abuse. With the growing popularity of kratom, efficient procedures are needed to detect kratom use. In the current study, both ultra-high-performance liquid chromatography and high-performance liquid chromatography–tandem mass spectrometry methods have been developed and validated for monitoring the major alkaloids and metabolites found in urine following kratom use. The primary unique alkaloid mitragynine is quantified in human urine from 1.00–500.00 ng/mL using mitraphylline as an internal standard. In addition, two metabolites (5-desmethylnitragynine and 17-desmethylhydromitragynine) and the related active, alkaloid 7-hydroxy-mitragynine, are simultaneously qualitatively monitored. The presence of analytes are confirmed by an information-dependent acquisition-enhanced product ion procedure generating full fragmentation data used to positively identify detected analytes. The validated method has been utilized for clinical and forensic analyses of urine for the detection of kratom use.

Introduction

The plant Mitragyna speciosa korth, or Kratom, is native to Thailand and other southeast Asian countries, where it has historically been viewed as a medicinal plant. Kratom leaves can be chewed, brewed into tea or smoked to produce a variety of psychoactive effects. Kratom is now considered a controlled substance in Australia and many southeast Asian countries (1). Awareness and use of kratom as an uncontrolled psychoactive drug has grown in the United States and European countries. The powdered plant material and purified kratom extracts are easily available through internet sites and herbal shops throughout the US (2). In the summer of 2011 the US Drug Enforcement Agency (DEA) declared kratom a drug of concern, but has not yet moved to classify the drug as a scheduled substance (2).

Kratom has been described as having both opium-like sedative and coca-like stimulant effects due to its dual binding to opioid and alpha adrenergic receptors (1, 3). Although there is no recognized medical use for kratom, several online forum posts and case studies suggest that it is being utilized for recreational use as a psychoactive drug, and to mitigate the effects of opiate withdrawal (4). Litttle is known regarding the acute or long-term effects of kratom use on human health. The generation of this data is partially hampered by a lack of effective methods for monitoring kratom use. For example, two case studies reported by Nelsen et al. and Boyer et al. describe situations in which individuals self-medicating with kratom to manage pain were admitted to the hospital following seizures (5, 6). Because kratom could not be detected in available toxicology screens, the attending physicians were only aware of the kratom use due to self reporting by the patients. These cases illuminate the need for methods that detect kratom use in emergency situations and that determine trends of kratom abuse, to identify the risks of long-term use.

Several unique alkaloids are present in kratom leaves; the predominant alkaloid is mitragynine (MG), followed by paynantheine, speciogynine and speciociliatine (7). None of the prevalent alkaloids appear to be structurally related to opiates or more typical drugs of abuse. MG, the most abundant alkaloid, has been investigated for its pharmacologic activity (7–9). Similar to morphine, MG has analgesic effects and is an opiate agonist binding to the μ-receptor, although it has lower affinity than morphine for the receptor (7, 9). Another less prevalent alkaloid of kratom, 7-hydroxy-mitragynine, has also been studied for analgesic activity and has been shown to be more active than both MG and morphine (10). The reported stimulatory action of kratom on the central nervous system and motor activity has not been studied as extensively as the analgesic effects. Several studies of rodents given MG via IP injection show signs of relaxation rather than motor stimulation (11). However, a study of paramecia, cats and humans did show signs of motor stimulation at lower MG doses (12). Similarly, users of kratom have reported feeling active and happy shortly following consumption (13). It is proposed that at low doses, MG binds alpha adrenergic receptors similar to the structurally related alkaloid, yohimbine, rather than to the μ and delta opioid receptors (3).

Several research groups have studied the metabolism of the kratom alkaloids to identify possible markers of kratom use, among other reasons. The major alkaloids of kratom, MG, paynantheine, speciogynine and speciociliatine, in addition to several metabolites, have been detected in urine of rats and humans following ingestion of kratom or its individual alkaloids (14–23). The first reported bioanalytical method for the quantitation of MG was a high-performance liquid chromatography–ultraviolet detection (HPLC–UV) method measuring MG in the serum of dosed rats, with a limit of quantitation (LOQ) of 100 ng/mL (19). More recently, mass spectrometry (MS) methods have been utilized to investigate the metabolism of kratom alkaloids in rats and humans (14–18) and a few bioanalytical MS methods have been reported (20–22). The goal for this project was to build upon the single quantitative LC–tandem mass spectrometry (MS-MS) method (20) and two
qualitative methods [an LC–MS-MS method (21) and a gas chromatography (GC)–MS-MS method (22)] reported in the literature for the screening of kratom alkaloids in human urine. Two of three predicate MS-based methods generate qualitative data; although this is acceptable for detecting use, the generation of quantitative data can aid significantly in furthering the scientific community’s understanding of patterns of kratom use. Similarly, two of the predicate procedures forgo the hydrolysis of conjugated metabolites (20–21). Without this procedure, sample processing time is greatly reduced; however, metabolism studies have shown that MG is highly conjugated in human urine (17). Thus, the hydrolysis of conjugates may be necessary to accurately assess excreted concentrations of MG. An additional intention was to create a simple quantitative analytical procedure that fully utilized available quadrupole linear ion trap technology to raise the level of confidence in assigned positive identifications beyond that of multiple reaction monitoring (MRM), MRM ion ratios or ion ratios from electron impact GC–MS ionization.

In the current study, both ultra-high-performance liquid chromatography (UHPLC) and HPLC–electrospray ionization (ESI)-MS-MS methods are described for the quantitative analysis of MG and qualitative analysis of its metabolites and 7-hydroxy-mitragynine in human urine for clinical toxicology monitoring of kratom use. The method employs an enzymatic hydrolysis and liquid–liquid extraction procedure before MRM and information-dependent–enhanced product ion (EPI) full fragmentation spectrum analysis of MG using mitraphylline as an internal standard (IS). Due to the lack of commercial high-purity reference standards for 7-hydroxy-mitragynine and the metabolites of MG at the time of method development, the assay is qualitative for these components. Information-dependent acquisition (IDA) procedures are employed on an AB Sciex QTrap 5500 to ensure positive identification of the detected targets; upon detection of an adequate signal from the monitored MRM transitions, the linear ion trap is activated and collects a full fragmentation spectrum of the selected precursor ion. The collected spectrum is then compared against a reference spectrum of known composition. Metabolite MS transitions and retention times were estimated from expected metabolite structures based on published metabolism studies (18, 20) and compared to analytes observed in a urine sample from a known kratom user. Structures were predicted using ACD/MS Fragmenter software (version 12.5, build 47652). The incorporation of IDA for the qualitative identification of MG metabolites and 7-hydroxy-mitragynine provides the method with a greater conformational certainty than previously published methods. Additionally, the described instrumental requirements three minutes for each injection; providing fast instrument cycle times.

Experimental

Reagents

MG (99% purity) and 7-hydroxy-mitragynine (80% purity) were obtained from Toronto Research Chemicals (Ontario, Canada), mitraphylline, for use as IS (97% purity), was purchased from Cerilliant (Round Rock, TX), β-glucoronidase *E. coli* was from Roche (Madison, WI), acetonitrile and methanol were from Burdick and Jackson (Muskegon, MI), and all other chemicals were procured from Sigma (St. Louis, MO).

Instrumentation

The analysis was developed for use on a Waters Acquity UHPLC system coupled to an Applied Biosystems Sciex QTrap 5500 quadrupole linear ion trap mass spectrometer (Foster City, CA). However, the method was also modified for use on a system comprised of an Applied Biosystems Sciex API 5000 triple quadrupole mass spectrometer connected to an Agilent 1100 HPLC and CTC PAL autosampler. The method variant utilizing the API 5000 forgoes IDA and the generation of full MS fragmentation data.

UPLC–ESI-MS-MS analysis

A Waters Acquity UPLC HSS T3 column (2.1 × 50 mm, 1.8 μm) maintained at 50°C was eluted with a gradient mobile phase of 10 mM ammonium acetate with 0.1% formic acid (A) and acetonitrile (B) at 500 μL/min. The mobile phase composition initiated at 10% B, was linearly increased to 40% B over 0.8 min, and was maintained at 40% for 0.5 min before linearly decreasing back to 10% B over 0.2 min. A re-equilibration time of 0.7 min elapsed before the initiation of subsequent injections. The mass spectrometer method consisted of an MRM experiment for the quantitation of MG, as described in Table I. Upon detection of a signal corresponding to MG, IDA–EPI experiments ensued. The IDA was triggered by the detection of 4,000 counts within the specified MRM. IDA consisted of an EPI experiment scanning for fragments of the detected precursor ranging from 90 to 600 Da at a rate of 1,000 Da/s, utilizing a fill time of 250 ms, and a step size of 0.12 Da. For reference, library fragmentation spectra were generated from a urine sample of a known kratom user. At least an 80% match between the generated spectra and the library spectra was required to be considered a positive match.

LC–ESI-MS-MS analysis

The UPLC method was modified for use on a traditional HPLC utilizing a Betasil C18 column (2.1 × 100 mm, 5 μm; Thermo Fisher) maintained at 50°C and eluted with a gradient mobile phase of 10 mM ammonium acetate with 0.1% formic acid (A) and acetonitrile (B) at 500 μL/min. The mobile phase composition initiated at 10% B, was linearly increased to 90% B over 0.5 min, and was then maintained at 90% B for 0.5 min before

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor m/z</th>
<th>Product m/z (primary)</th>
<th>Collision energy, eV (primary)</th>
<th>Product m/z (secondary)</th>
<th>Collision energy, eV (secondary)</th>
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<tr>
<td>MG</td>
<td>399.3</td>
<td>174.1</td>
<td>28</td>
<td>238.1</td>
<td>26</td>
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<tr>
<td>7-OH-MG</td>
<td>416.3</td>
<td>191.0</td>
<td>33</td>
<td>238.1</td>
<td>26</td>
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<tr>
<td>5-OH-MG</td>
<td>385.3</td>
<td>174.1</td>
<td>33</td>
<td>238.0</td>
<td>26</td>
</tr>
<tr>
<td>17-OH-DMG-MG</td>
<td>367.1</td>
<td>174.1</td>
<td>33</td>
<td>238.0</td>
<td>26</td>
</tr>
<tr>
<td>Mitraphylline (IS)</td>
<td>359.3</td>
<td>159.9</td>
<td>33</td>
<td>309.2</td>
<td>23</td>
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</table>

Table I: Mass Spectrometer Transitions Monitored for MG, MG Metabolites, 7-OH-MG and the IS (Mitraphylline)
being linearly decreased back to 10% B over 0.5 min. A re-equilibration time of 2.3 min was required before the next injection. The mass spectrometer was operated in positive MRM mode monitoring for MG, 7-hydroxy-mitragynine (7-OH-MG), 5-desmethyl-mitragynine (5-DM-MG), 17-desmethyl-dihydromitragynine (17-DMD-MG) and mitraphylline. The monitored transitions and collision energies are displayed in Table I; the dwell time was 30 ms for each transition. Other mass spectrometer parameters were as follows: curtain gas, 38 psi; ion spray, 3800 V; temperature, 500°C; gas 1 and 2, both 63; declustering potential, 60 V; entrance potential, 10 V; and exit potential, 19 V. The quantitation of MG was performed from the ratio of MG to mitraphylline peak areas and a 1/concentration² linear regression.

Preparation of quantitative standards and controls
Stocks were prepared in methanol (0.25 mg/mL MG and 1.0 mg/mL mitraphylline) and diluted with methanol–water for a working IS of 1 µg/mL and MG concentrations of 2, 0.4, 0.04, 0.02, 0.01 and 0.004 µg/mL. Stocks and working standards were stored at 4°C under mostly dark conditions when not in use. Quality control (QC) samples included three quantitative positive controls and three qualitative controls. Quantitative controls were prepared by spiking blank matrix with MG to final concentrations of 3.00, 75.00 and 400.00 ng/mL. Quantitative controls were considered acceptable if with 20% of theoretical with a relative standard deviation (RSD) of replicate determinations of less than 20%. The qualitative controls included a negative blank urine control and a positive control of blank urine fortified with both MG and 7-OH-MG at 100.00 ng/mL each, and a positive sample containing 7-OH-MG and MG and metabolites from a known kratom user. Retention time and purity matching criteria were applied to all positive qualitative controls, and the negative qualitative control was required to be free of all detected analyte signal in excess of 50% of the lower limit of quantitation (LLOQ).

Sample hydrolysis
Before sample extraction and analysis, phase II metabolic conjugates were enzymatically hydrolyzed. Urine sample aliquots (200 µL) were fortified with 25 µL of IS solution and then diluted with 200 µL potassium phosphate hydrolysis buffer (50 mM, pH 6). β-Glucuronidase was then added at sufficient quantity to achieve an enzyme activity of 20,000 units per sample. Samples were then briefly mixed and incubated at 38°C for 3 h. Following hydrolysis, samples were allowed to equilibrate to room temperature before alkalization by the addition of 500 µL of 0.1 M NaOH. Samples were then processed by liquid–liquid extraction.

Liquid–liquid extraction
Methyl tert-butyl ether (MTBE, 3 mL) was added to each tube and vortex-mixed for 5 min. Samples were then centrifuged for 5 min at approximately 1,000 G to separate the organic and aqueous layers. The samples were then placed in a dry-ice/IPA bath to freeze the aqueous layer, and the organic extract was decanted into a glass conical tube and dried under nitrogen at 45°C. The residue was reconstituted with 500 µL of methanol before instrumental analysis, as described previously.

Method validation
The quantitative part of the method was validated using the following experiments: an assessment of the performance of assay calibrators, intra-run and inter-run precision and accuracy, recovery, selectivity, room temperature stability, refrigerated stability, freeze-thaw stability, frozen stability, processed batch stability, and analysis of the applicability of dilutions, carry-over and interference were performed in general accordance with the Guidance for Industry: Bioanalytical Method Validation (24). With the exception of assay selectivity, method validation criteria were solely applied to the quantitation of MG.

Assay calibration
The quantitative part of the assay utilized a six-concentration standard curve ranging from 1.00–500.00 ng of MG per mL of human urine. The calibrators were prepared by spiking 0.2 mL of urine with standards to final concentrations of 1.00, 2.50, 5.00, 10.00, 100.00 and 500.00 ng/mL. Following the addition of the IS (125 ng/mL) the calibrators were extracted and analyzed by the UPLC–ESI-MS-MS and LC–ESI-MS-MS methods described above. Quantitation of MG was performed using mitraphylline as an IS and a 1/x² linear calibration using Analyst 1.4.2 or 1.5.1 software. Acceptance criteria applied to the curve were ±20% RSD for each point.

Accuracy and precision
The accuracy and precision of the assay was evaluated by analyzing five replicates of low, medium, high and LLOQ QCs on three separate days. QCs at each concentration were required to average within 20% of theoretical, with a precision of within 20% RSD. Additionally, LLOQ controls were required to possess a signal-to-noise ratio in excess of 5.

Recovery
The recovery of MG and mitraphylline were determined by comparing the detected MRM peak areas from inter-run QC samples to the signal strength of fortified solvent standards at a concentration equivalent to a theoretical recovery of 100%. No acceptance criteria were applied to recovery; however, recovery of the analyte was consistent, concentration-independent and similar to the recovery of the IS.

Selectivity
Ten random urine samples were analyzed as blank samples. Aliquots of the same samples were then fortified with two concentrations of MG: 1.0 and 75.0 ng/mL. The assay was considered selective if the unfortified samples contained no detected peaks for any of the monitored analytes, and no IS peak exceeding 5% of the average IS peak area is observed in the calibration curve. Furthermore, fortified samples were required to quantify to within 25% of theoretical at LLOQ and within 20% of theoretical at concentrations in excess of LLOQ.
Suppression

The potential for the assay to be impacted by ion suppression or ion enhancement was evaluated using the same random urine samples from the selectivity experiment using the procedure first detailed by Matuszewski et al. (25). The samples were extracted as unfortified blank samples and carried through the entire extraction process. The final residue was then fortified with MG and IS to a concentration equivalent to an LLOQ concentration. The raw MG and IS responses from each sample were then compared to solvent standards of matching concentrations. Analyte and IS matrix factors were determined by dividing the analyte response from the fortified matrices by the analyte response from the solvent standard. A normalized matrix factor was then calculated by dividing the analyte matrix factor by the IS matrix factor.

Stability

For stability experiments, a minimum of four replicates of the low and high QCs were stored at ambient conditions, 4°C and –20°C for five days. Freeze-thaw stability was investigated for four replicates cycled through three freeze-thaws. The acceptance criterion for all stability samples was identical to that of inter-run accuracy and precision QC performance.

Dilution

The ability to dilute over-range samples was investigated by fortifying control urine with MG to a concentration of 1,600 ng/mL. This sample was then analyzed neat and following 5-fold and 10-fold dilutions with blank urine. Dilution was acceptable if the diluted samples quantified to within 20% of the nominal value.

Carryover

The potential for carryover to impact the assay was investigated throughout the validation. A blank sample was injected following every upper limit of quantitation (ULOQ) calibrator. Acceptable limits for matrix blanks following these samples was a peak area less than 50% of the area of the concurrently analyzed LLOQ standard. Additionally, carryover was investigated by analyzing a blank sample following the neat injection of the pre-diluted, over-range QC (containing 1,600 ng/mL of MG). The same carryover acceptance criteria were applied.

Interferences

The potential for common drugs of abuse and over-the-counter medicines to interfere with the assay was investigated by spiking matrix blanks and medium concentration QC samples with 500 ng/mL of each investigated drug (Table II). Similar to selectivity criteria, interference blanks were required to contain an analyte signal of no more than 20% of the response at LLOQ, and the accuracy of QC samples containing the potentially interfering compounds was required to quantify within 20% of the nominal value.

Results and Discussion

A sensitive and specific method has been developed and validated for the detection of kratom alkaloids in urine samples. Although kratom is not currently regulated in the United States or many European countries, it is on the DEA list of drugs of concern due to its growing popularity, ease of availability and lack of knowledge regarding its long-term effects on health (2). The assay is a combination of qualitative and quantitative procedures incorporating both MRM data and information-dependent EPI spectra. The assay has been successfully validated generating the validation data described in the following.

Chromatography and mass spectrometry

No interfering peaks were observed in blank control urine, and the signal strength at LLOQ (1.00 ng/mL) was acceptable, with an average signal-to-noise ratio of 596. The choice of monitored species for the qualitative aspect of the assay was based upon published reports of MG metabolite structures (18, 20). Expected metabolites were confirmed by utilizing urine obtained from a known kratom user (Figure 1). Observed product spectra for MG and 7-OH-MG reference standards were consistent with the theoretical fragmentation of the alkaloids (Figures 2A–B). Due to a lack of reference standards for the metabolites of MG, spectra and observed peak retention times obtained with a known positive sample were used to create a library of metabolites for future kratom confirmation studies (Figures 2C–D).

Table II

<table>
<thead>
<tr>
<th>Over-the-counter</th>
<th>Drugs of abuse</th>
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<tbody>
<tr>
<td>Acetaminophen</td>
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</tr>
<tr>
<td>Ibuprofen</td>
<td>Carboxy-tetrahydrocannabinol</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Cocaine</td>
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<tr>
<td>Ketoprofen</td>
<td>Benzoylhexagonine</td>
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<tr>
<td>Pseudoephedrine</td>
<td>Codeine</td>
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<tr>
<td>Phenytoin</td>
<td>Morphine</td>
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<td>Phenylephrine</td>
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<td>Oxymetazoline</td>
<td>Diazepam</td>
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<td>Guafenesin</td>
<td>Nordoxepin</td>
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<tr>
<td>Dextromethorphan</td>
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<td>3,4-Methylenedioxyamphetamine</td>
</tr>
<tr>
<td>Hydroxyzine</td>
<td>Oxycodone</td>
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Assay calibration

The assay was calibrated using six-point calibration curves with mitraphylline as an IS and 1/x² linear regression. The calibration curves were linear over the range of 1.00–500.00 ng/mL. For four independent curves analyzed during the validation, a mean and standard deviation coefficient of variation (CV) of 0.9972 ± 0.0018 was obtained. The assay range of 1 to 500 ng/mL was intended to be consistent with the results of Lu et al. (20), who reported 167 ng/mL of MG in the urine of a known kratom user.
Accuracy and precision
Intra-run accuracy and precision were determined by analyzing five replicates of fortified controls at MG concentrations of 1, 3, 75 and 400 ng/mL on a single batch. The mean concentrations were within 15% of the target for each concentration, and precision ranged from 3.7–9.9% (Table III). The inter-assay accuracy and precision were determined by analyzing the same controls on three separate batches, each utilizing replicates of five. The mean concentrations were within 14% of the target value for each QC, and precision ranged from 6.7–15.4% (Table III). All were considered acceptable for the intended use of the assay.

Recovery
The recovery of MG and mitraphylline from urine was determined at three concentrations: 3.00, 75.00 and 400.00 ng/mL. Recoveries were determined by comparing two sets of samples: standards fortified into urine and analyzed after processing as samples, and standards fortified into solvent analyzed as injection standards. MG recovery ranged from 77.8–94.0% with a mean of 85.0%, and the mitraphylline recovery averaged 80.0%. The relatively similar recovery for analyte and IS is of significance because isotopically labeled ISs are not currently available.

Selectivity
The specificity of the method was determined by analyzing 10 randomly selected urine samples as blanks, and after fortifying the samples with MG to concentrations equivalent to both the LLOQ of the assay and a medium concentration control. No MG/mitraphylline peaks were detected in specificity blanks in excess of 20% of LLOQ. All fortified samples quantified within the acceptance limits, except for a single sample fortified to LLOQ, which quantified at 126% of the target value. The mean of all LLOQ fortified samples, however, was within 16% of the target concentration (Table IV), and the selectivity of the assay was acceptable.

Suppression/matrix effects
Ionization suppression and enhancement were monitored by extracting 10 random urine samples as blanks, and then fortifying with MG/mitraphylline to a concentration equivalent to
Figure 2. Spectra of the studied compounds: MG reference standard (A); 7-OH-MG reference standard (B); 5-DM-MG (C); 17-DMD-MG (D).
LLOQ in the reconstitution solvent. These samples were compared to solvent injection standards at matching concentrations. Matrix factors for MG were normalized to the mitraphylline matrix factor. The normalized matrix factor ranged from 0.965 – 1.308 with a CV of 8.7%, indicating a lack of significant, uncontrolled matrix effects.
The stability of MG in urine using two QC concentrations (3.00 and 400.00 ng/mL) was investigated following three freeze-thaw cycles. Stability was also evaluated following five days of storage at refrigerated, frozen (−20°C) and ambient conditions. MG was accurately quantified following all storage conditions, as reported in Table V. It was thus concluded that samples may be accurately assayed for kratom use following typical sample shipping and storage conditions.

Carryover
The potential impact of carryover was monitored by analyzing blanks following ULOQ standards and following an over-range sample (four times the concentration of the high QC). No carryover was observed in blanks following ULOQ standards; however, carryover was present in the blank sample that followed the over-range sample at 82% of LLOQ. Therefore, in situations in which over-range samples are present, the subsequent sample must be reanalyzed due to the potential impact of carryover.

Interferences
To investigate interference to the assay from common over-the-counter drugs and drugs of abuse, blank urine and medium QCs were fortified with 500 ng/mL of the drugs listed in Table II. No MG signal was detected in the blanks, and the fortified QC samples quantified within 10% of the target concentration (data not shown). Therefore, there was no interference from the investigated drugs.

Dilutions
The acceptability of the method to measure dilutions of over-range samples or samples of insufficient volume was investigated by preparing a QC at 1,600 ng/mL and diluting with blank matrix five-fold and 10-fold in triplicate before extraction. Dilution of over-range samples was deemed acceptable, with accuracy and precision at 83.4 ± 6.2% and 92.3 ± 0.0% of the target value for five-fold and 10-fold dilutions, respectively.

Application to forensic/clinical analysis
Following assay validation, the methodology was placed into general use. Urine samples positive for kratom use were identified using the validated procedure. MG was detected and quantified using MRM transitions of 399.3→174.1 and 399.3→238.1 (Figure 1) and IDA–EPI-based procedures. Furthermore, MG, MG metabolites (5-DM-MG and 17-DMD-MG) and 7-OH-MG were also detected and confirmed using IDA–EPI-based procedures. Most positive samples originated from drug court investigations. Urine MG concentrations ranged from 1.2 ng/mL to greater than 50,000 ng/mL. Figure 3 shows a histogram of measured MG concentrations from 50 positive samples. To date, all samples identified as containing MG also contained 7-OH-MG. Unfortunately, no history of use could be obtained from these donors. Levels of the monitored metabolites were smaller and more variable than those of MG. Comparative 7-OH-MG and metabolite concentrations based on the raw MG response from 12 randomly selected positive samples are shown in Table VI.

Post-validation investigations
Following assay validation and the accumulation of a significant number of positive samples, specific method parameters were re-evaluated. First, the necessity of enzymatic hydrolysis was investigated. Positive samples possessing MG concentrations between 4 and 6,170 ng/mL were processed with the hydrolysis procedure omitted. Results from this limited sample set indicated that at MG concentrations of less than approximately 1 μg/mL, most of MG is excreted without conjugation. Only at concentrations in excess of 1 μg/mL did the conjugated metabolites play a significant role, and even then, they accounted...
A specific and selective LC-MS-MS assay has been developed for detecting the use of kratom in urine samples. The assay utilizes a quantitative MRM procedure for the dominant kratom alkaloid, MG. Additionally, a qualitative information-dependent procedure is utilized to identify 7-OH-MG and MG metabolites. The qualitative method compares the full fragmentation spectra of detected species against those generated from a known kratom user. The use of additional conformational data adds a significant level of assurance against false negative data for no more than 50% of the excreted MG. Results imply that depending on the purpose of testing, the hydrolysis procedure may be skipped to improve the speed of the analysis, but high MG concentrations may be underestimated.

Additionally, collected positive samples allowed for the further evaluation of the choice of IS. Mitraphylline is structurally related to MG and performs well as an MG IS. However, mitraphylline is reported to be a minor constituent of kratom (26), and thus may be present in the urine of kratom users. The assay was originally developed with the aid of a single positive sample that was shown to not possess any detectible mitraphylline before IS fortification. More recently, 10 additional positive samples (ranging in MG concentrations from 4 to 6,170 ng/mL) were evaluated without IS fortification, and were then processed and monitored to detect the presence of any mitraphylline. In unfortified samples, mitraphylline was either undetected or detected at concentrations less than 2% of the IS fortification, which is too small to detrimentally impact the accuracy of the quantitation. Thus, post-validation analysis of positive samples reaffirms the acceptance of mitraphylline as a quantitative IS for kratom analysis.

Conclusions

A specific and selective LC–MS-MS assay has been developed for detecting the use of kratom in urine samples. The assay utilizes a quantitative MRM procedure for the dominant kratom alkaloid, MG. Additionally, a qualitative information-dependent procedure is utilized to identify 7-OH-MG and MG metabolites. The qualitative method compares the full fragmentation spectra of detected species against those generated from a known kratom user. The use of additional conformational data adds a significant level of assurance against false negative data beyond previous studies by new methods (20–22). Positive samples have been identified with MG concentrations ranging from 1 ng/mL to greater than 50 μg/mL.


