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Quantitative analysis of mitragynine in human urine by high performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

Mitragynine is the primary active alkaloid extracted from the leaves of *Mitragyna speciosa* Korth, a plant that originates in South-East Asia and is commonly known as kratom in Thailand. Kratom has been used for many centuries for their medicinal and psychoactive qualities, which are comparable to that of opiate-based drugs. Kratom abuse can lead to a detectable content of mitragynine residue in urine. Ultra trace amount of mitragynine in human urine was determined by a high performance liquid chromatography coupled to an electrospray tandem mass spectrometry (HPLC-ESI/MS/MS). Mitragynine was extracted by methyl t-butyl ether (MTBE) and separated on a HILIC column. The ESI/MS/MS was accomplished using a triple quadrupole mass spectrometer in positive ion detection and multiple reactions monitoring (MRM) mode. Ajmalicine, a mitragynine's structure analog was selected as internal standard (IS) for method development. Quality control (QC) performed at three levels 0.1, 1 and 5 ng/ml of mitragynine in urine gave mean recoveries of 90, 109, and 98% with average relative standard deviation of 22, 12 and 16%, respectively. The regression linearity of mitragynine calibration ranged from 0.01 to 5.0 ng/ml was achieved with correlation coefficient greater than 0.995. A detection limit of 0.02 ng/ml and high precision data within-day and between days analysis were obtained.

1. Introduction

Mitragynine, chemically known as 9-methoxy-corynantheidine, the primary active alkaloid in the plant *Mitragyna speciosa* Korth, which is commonly known as kratom in Thailand [1]. Leaves of kratom have been used by the natives of Southeastern Asian countries, including Thailand, Malaysia, and Myanmar since the early 19th century, because of the plant's analgesic and stimulant effects [2,3]. Kratom has been also used as a substitute for opium and for withdrawal treatment of morphine addicts [4]. The chemical structures and pharmacological properties of mitragynine and its analogues have been much studied [2,4–6]. As well as narcotic, analgesic, and stimulant-like effects, due to agonistic action on opioid receptors [2,7–9], mitragynine and its analogues have been shown to have inhibitory effects on neurogenic systems, in animal experiments [10–12].

Despite these interesting pharmacological properties, little work has been done on the analysis of mitragynine and its analogues in human specimens. The current widespread availability of kratom on the Internet reflects the extensive demand for this product in the world, and is indicative of an emerging trend to use kratom as a non-controlled drug substance to replace morphine-like drugs, since kratom usage is not monitored by most of the national drug abuse surveys including U.S. [3,13].

An emergency biomonitoring method for detecting of the use of this substance was requested by New York State Poison Control Center for a patient found unresponsive after using this drug. This study describes the development of a suitable bioanalytical method to quantify trace amounts of mitragynine in human urine specimens by HPLC-MS/MS. Several natural substances were evaluated as potential internal standard (IS) for the method development. The typical isotopically labeled analog to the target compound would be optimal choice; however, since no isotopically labeled mitragynine is commercially available, ajmalicine, a structural analog to mitragynine, was selected as IS. Blank urine was used as the matrix throughout the method development. Liquid extraction of the urine spiked with mitragynine was carried out, and several organic solvents, such as ethyl ether, ethyl acetate, and methyl t-butyl ether (MTBE) were evaluated. The quality control (QC) requirements include the demonstration of low system background, high accuracy, and high precision; acceptance criteria were also established for the method development.

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2. Experimental

2.1. Reagents

The raw kratom leaves powder which was used for mitragynine standard preparation, was purchased from an Internet store as 15× raw leaf extract. Ajmalicine ($C_{21}H_{24}N_2O_3$, purity 99%), anhydrous di-sodium hydrogen orthophosphate, acetic acid 99.8%, ammonium acetate 99.99%, and ammonium hydroxide A.C.S. reagent were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). All solvents used were HPLC grade or better, and were supplied by Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). SilicAR 60 Å silica-gel was obtained from Mallinckrodt Baker (Paris, KY, USA). Purified water was prepared in the laboratory with a Nanopure Diamond water system (Barnstead International Inc., Dubuque, IA, USA).

2.2. LC-MS/MS instrument

The HPLC-MS/MS system consisted of an Agilent Technologies 1200 Series HPLC (Wilmington, DE, USA) equipped with a vacuum degasser, a binary pump, a thermostatted column compartment, and an autosampler. The HPLC system was coupled to an API-2000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada), which was equipped with a turbo electrospray ionization (ESI) source, operated in positive mode using nitrogen as nebulizer gas. The ESI sheath gas was set at 50 psi. The operating MS/MS parameters are detailed in Section 3.1. HPLC-MS/MS data were collected and processed by Analyst software, version 1.4.2 (Applied BioSystems/MDS SCIEX).

HPLC separation was performed on a silica-gel based Waters (Milford, MA, USA) 50 mm × 3.0 mm (length × i.d.), 3 μm (particle size) Atlantis HILIC column thermostatted at 40 °C; it used mobile phase A, consisting of 5 mM ammonium acetate, and mobile phase B consisting of methanol, at a flow rate of 0.25 ml/min. A sample volume of 10 μl was injected onto the HPLC column. Separation was carried out according to the following gradient program: 0–3 min, 90% B to 100% B; 3.1–7 min, and 90% B.

2.3. Purification of mitragynine

Mitragynine standard used in this study was extracted and purified in our laboratory from raw kratom, since no mitragynine standard is commercially available. The process of isolation and purification of mitragynine was previously described by Houghton et al. [14], Ponglux et al. [15], and Janchawee et al. [16]. Briefly, an aqueous suspension of kratom raw leaf extract (5 g in 100 ml 10% acetic acid), was filtered through filter paper, and then extracted with 100 ml of *n*-hexane. The aqueous solution was adjusted to pH 9 with 25% aqueous ammonium hydroxide solution, and extracted again with *n*-hexane and then dichloromethane. The extracts were washed using deionized water, and then dried with sodium sulfate anhydrous. Solvent was removed with a rotary evaporator until dryness. An aliquot of residue (1 g) in dichloromethane was subjected twice to silica-gel column chromatography, and was eluted with 10% methanol in chloroform. The eluate was evaporated to dry and recrystallized with methanol and chloroform, to yield a major alkaloid (0.1 g), which appeared as a single spot on a silica thin layer chromatography (TLC) analysis using ethyl acetate/methanol (4/1, v/v) as mobile phase. In general, 10 g of 15× kratom raw leaf extract yields approximately 0.5 g of purified mitragynine (about 5% yield based on the crude extract weight).

Purified mitragynine was found to produce a predominant single chromatographic peak upon spectroscopic analysis by an Agilent 6972/5972 gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies Inc., Wilmington, DE, USA), operated in electron impact (EI) with scan mode (100–500 *m*/*z*) using ChemStation Version A 3.00 software (Agilent Technologies Inc.) The spectrum of purified mitragynine ($C_{23}H_{30}N_2O_4$, exact molecular mass = 398.2207) was confirmed by comparison to the NIST 98 mass spectral library. Mitragynine obtained from this procedure was approximately 95% pure, based on comparison the peak area of mitragynine to other minor impurities from GC-MS analysis, and was used as the analytical standard throughout the method development.

2.4. Preparation of standard solutions

Stock solutions of 500 μg/ml of mitragynine and 100 μg/ml of ajmalicine (IS) were prepared by dissolution of standard compounds in 100% methanol. The stock solution was further diluted with methanol for preparation of working standard solutions at lower concentrations. All solutions were stored at −20 °C, and were thawed and vortex mixed at room temperature before use. The stock solutions are stable during a study period of 60 days.

2.5. Sample extraction

Both pooled blank and patient urine samples were stored at −80 °C until analysis. Urine samples were thawed and 2.0 ml aliquot was transferred to 10 ml clear glass screw-top culture tube (Kimax, Veniland, NJ, USA) and spiked with 20 μl of 100 ng/ml IS working solution. 500 μl of Na_2HPO_4 buffer (0.5 M aqueous, which had been adjusted to pH 11 with 25% aqueous sodium hydroxide) was added to each urine sample, and the mixture was vortexed for 30 s. A 3 ml of MTBE was further added to the samples, which was then shaken vigorously on an Eberbach 6000 reciprocating shaker (Ann Arbor, MI, USA) for 20 min, and centrifuged in an Eppendorf 5804 centrifuge (Hamburg, Germany) at 1000 × *g* for 10 min. The supernatant was then transferred to another clean tube and evaporated to dryness under a gentle nitrogen stream at 45 °C, using a Zymark Turbovap LV concentrator (Hopkinton, MA, USA). The extract was finally reconstituted in 1 ml of methanol and transferred to a sample vial for HPLC-MS/MS analysis.

2.6. Calibration

Quantitation of mitragynine was calibrated by IS technique, using Analyst software. The calibration curve was constructed by plotting the ratios of the peak area of mitragynine and IS against the ratios of concentration of mitragynine and IS. Regression analysis with weighting factor of $1/x$ was conducted to obtain the calibration equation and correlation coefficient (r). Linearity was evaluated through preparation of seven standard concentrations of mitragynine in blank urine samples at the concentrations of 0.01, 0.025, 0.05, 0.2, 1, 2.5, and 5.0 ng/ml. The IS was added and the calibration samples were then extracted as described in Section 2.5, before HPLC-MS/MS analysis. Established acceptance limits for calibration curve of mitragynine were ±20% for relative standard deviation (RSD) for all response factors, and correlation coefficient of 0.99 or greater.

2.7. Method development and quality control

Method development was performed in accordance with the Guidance for Industry: Bioanalytical Method Validation [17]. Pooled blank urine, which was free of mitragynine, was mixed and filtered through grade 3 filter paper (Whatman International Ltd., Springfield Mill, UK), and used as sample matrix throughout the method development.

The QC samples include three matrix samples, which were spiked with mitragynine at 0.1, 1.0 and 5.0 ng/ml, and one matrix blank. The matrix spike samples were stored at −80 °C until use.

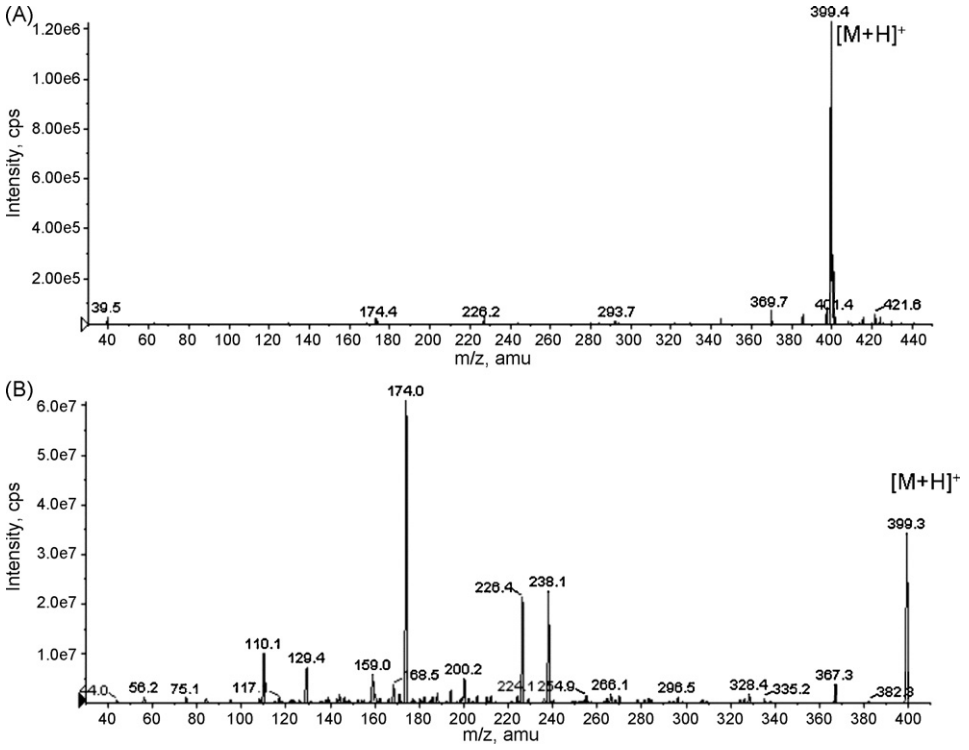

Fig. 1. Mass spectra of mitragynine. (A) Positive ESI in full-scan mode, and (B) in transaction of $[M+H]^+$ m/z 399 product-ion scan mode acquired at collision energy of 40 eV.

The precision, expressed as a relative standard deviation (RSD), and the recoveries (quantified value/spiked value) were evaluated, within-day, by determination of mitragynine amounts in three spiked levels cited above. Inter-day precision was determined by daily assay of the samples at four timepoints: when fresh, after 1 day, 7 days, and 28 days of storage at −80 °C. Accuracy was acceptable when the recovery was within ±30% of the nominal concentration. The acceptance criterion for precision was an RSD value within ±20%.

The method detection limit (MDL) was set at three times the standard deviation of the calculated concentration above the mean concentration, as determined in seven replicates of the matrix spike at 0.025 ng/ml mitragynine. The lower limit of quantification (LLOQ) of mitragynine was set at five times of the MDL.

3. Results and discussion

3.1. MS/MS optimization

The operating parameters for the ESI source were optimized to obtain the best mass spectrometric performance for both mitragynine and ajmalicine. The molecular ion and product ions of mitragynine and ajmalicine were observed through continuous infusion of each compound at the concentration of 1 μg/ml in methanol, with the ESI source operating in both positive and negative modes. Full-scan mass spectra were recorded from 35 to 450 amu for evaluation of the performance of the signal in each ionization mode. The signal intensities obtained in positive mode were found to be much higher than those in negative mode, for both mitragynine and ajmalicine. Full-scan daughter mass spectra were also evaluated, with continuous infusion in product-ion scan mode. The most abundant product ion for each compound was selected for MS/MS quantification.

The full-scan mass spectrum of mitragynine showed a strong signal for the ion at m/z = 399 corresponding to the protonated molecular ion $[M+H]^+$ (Fig. 1A). Few ion fragments observed in the full-scan mass spectrum of mitragynine indicate that the molecular ion is very stable. The product ion spectrum of mitragynine showed abundant daughter ions at m/z = 174, 226, and 238 generated at a collision energy (CE) of 40 eV (Fig. 1B), suggesting that the precursor ion m/z = 399 is readily converted to the product ions m/z = 174, 226 and 238 under collisionally activated dissociation (CAD) conditions. The masses of mitragynine product ions (daughter ions) were measured and their corresponding empirical formula was calculated using the Analyst software, version 1.4.2 (Applied BioSystem/MDS Sciex). The fragment patterns of mitragynine under CAD conditions are shown in Fig. 2. The most abundant daughter ion at m/z 174, is a good candidate to monitor the quantitative transition of m/z 399 > 174 during the CAD reaction using MRM experiment, while the multiple second transitions of m/z 399 > 226 and m/z 399 > 238 can be used as confirmation ions, which are the only characteristics of mitragynine.

The full-scan and product-ion scan mode spectra of the ajmalicine exhibited the precursor ion at m/z 353, corresponding to compound's protonated molecular ion $[M+H]^+$ (Fig. 3A), and the ion at m/z 144 as the most abundant daughter ion obtained at collision energy of 30 eV (Figs. 3B and Fig. 4). No other important fragments were observed for this compound. Therefore, the transition m/z 353 > 144 was chosen for quantification of ajmalicine in the MS/MS experiment.

MS/MS parameters, include ionization energies, temperature, and the voltages applied to the ESI source, were optimized in MRM mode for both mitragynine and ajmalicine infused at 1 μg/ml in 90:10 of methanol:ammonium acetate (5 mM) at 0.25 ml/min using flow injection analysis (FIA) optimization (Table 1). For optimum MS/MS precision, there must be at least 20 scans across the peak. The optimization of the flow rates of nebulizing gas, auxiliary gas, and curtain gas was also performed by FIA to maximize the sensitivity of the MRM signals for mitragynine and ajmalicine. The quantitation of mitragynine was performed in MRM mode with three mass transitions of m/z 399 > 174, 399 > 226, and 399 > 238

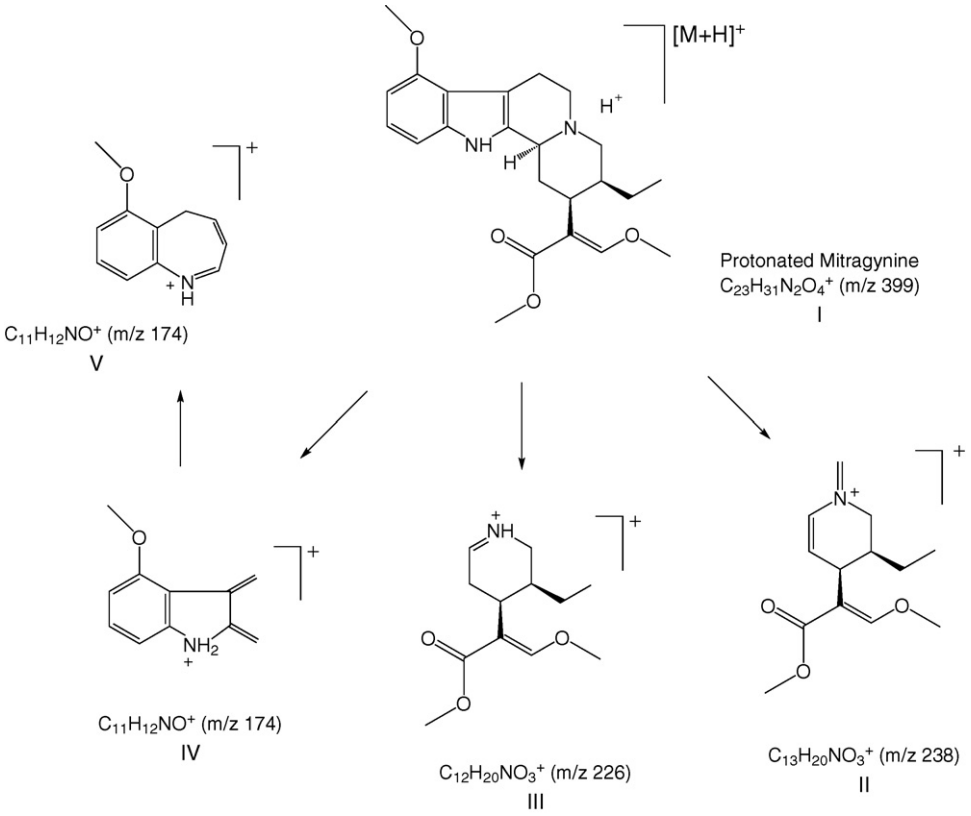

Fig. 2. Chemical structure of protonated mitragynine (I) and tentative identification of its fragment patterns (II, III, IV, and V) under CAD conditions. The structure analog to V was suggested by Khmel'nitskii [18].

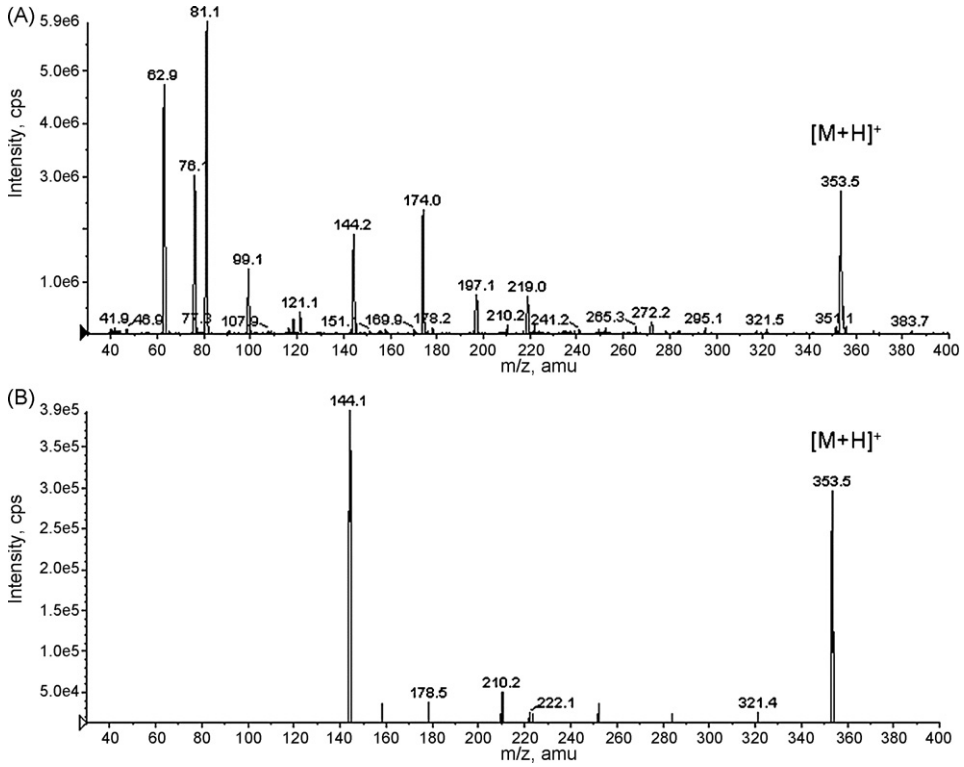

Fig. 3. Mass spectra of ajmalicine. (A) Positive ESI in full-scan mode, and (B) in transaction of $[M+H]^+$ m/z 353 product-ion scan mode acquired at collision energy of 30 eV (B).

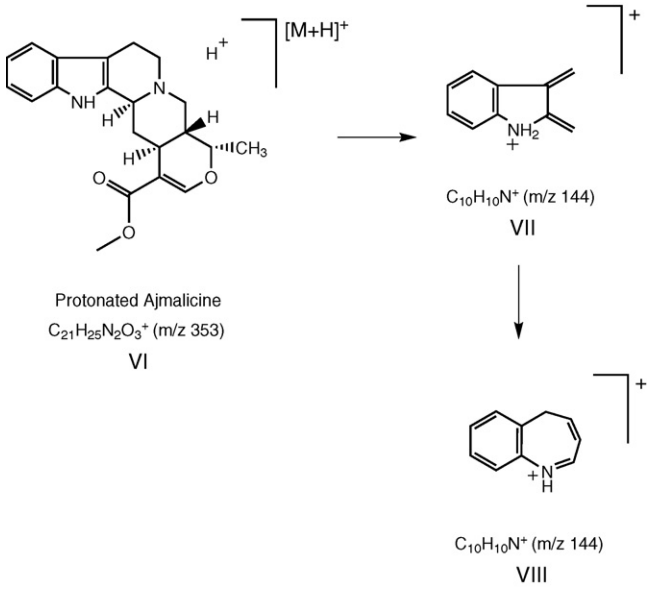

Fig. 4. Chemical structure of protonated ajmalicine (VI) and tentative identification of its fragment patterns (VII, and VIII) under CAD conditions. The structure of VIII was suggested by Khmel'nitskii [18].

to enhance the signal sensitivity, while only the transition *m*/*z* 353 > 144 was used for ajmalicine. The dwell time was set at 800 ms for both compounds.

3.2. LC analysis

The HPLC separation of mitragynine and ajmalicine was initially performed on the Agilent Zorbax Extend-C18 column (4.6 mm × 150 mm i.d. × length; 3.5 μm particle size) using methanol and 5 mM ammonium acetate as binary eluent solvents. The MS/MS operating parameters obtained in MRM mode described in the section above were applied for the quantification of mitragynine and ajmalicine. As previously reported by Janchawee et al. [16], mitragynine is insoluble in water but freely soluble in methanol. We found both mitragynine and ajmalicine to be readily soluble in a 90:10 mixture of methanol and 5 mM ammonium acetate. However, due to their strong hydrophobicity, the LC separation of these two compounds was not completely achievable on the C18 column: when a lower percentage of methanol was used, the mitragynine peak shape became broader and lower intense, while at a higher percentage of methanol, the two compounds almost co-eluted. On the other hand, the LC separation of mitragynine and ajmalicine is also dependent on the pH of the eluent: loss of mitragynine can occur due to hydrolysis of the compound at high pH.

A polar silica-gel based on stationary phase was used for this study. The separation of mitragynine and ajmalicine was achieved with a Waters Atlantis HILIC column (50 mm × 3.0 mm length × i.d.; 3 μm particle size) eluted in a 90:10 mixture of methanol and 5 mM ammonium acetate. A matrix standard, which contained both mitragynine and the ajmalicine in blank urine, was tested under the same conditions to reveal matrix effects and possible interferences. A typical matrix standard chromatogram is shown in the Fig. 5A and B. No interference was observed in the analyte or IS retention windows. The retention times for mitragynine and ajmalicine were 2.4 and 1.2 min, respectively. The separation of mitragynine and ajmalicine on HILIC column was also observed at three different temperatures of 30, 40 and 50 °C. Since no significant improvement was obtained, the column was thermostatted at 40 °C in order to reduce the LC pump back pressure.

Table 1
Optimized MS/MS operating parameters for mitragynine and ajmalicine obtained from API 2000 tandem mass spectrometry.

MS/MS parameter	Mitragynine	Ajmalicine
Polarity	Positive	Positive
Precursor ion (*m*/*z*)	399	353
Product ion (*m*/*z*)	174, 226, 238	144
Collision energy (eV)	45	40
Declustering potential (V)	50	50
Ionspray voltage (V)	4500	4500
Ion source temperature (°C)	550	550

3.3. Evaluation of liquid extraction

Urine has long been used for drug abuse testing, because the concentrations of metabolites and/or parent drugs are usually higher in this matrix than in other biological matrices, such as blood or oral fluid, and because larger volumes are readily available for collection [19]. Liquid–liquid extraction is widely used for sample clean-up.

We evaluated mitragynine liquid–liquid extraction with three solvents, ethyl acetate, ethyl ether and MTBE. Recoveries, defined as the ratio between quantified value and control spiked value, were determined using four replicate pooled urine samples, each spiked with mitragynine and ajmalicine at 1 ng/ml, and processed as samples. The control samples are the blank urine extracts, which were added, with the mitragynine and ajmalicine standard, just before injection.

The recoveries of mitragynine extracted by ethyl acetate, ethyl ether, and MTBE were 49, 82, and 81%, respectively (Table 2). Thus, ethyl ether and MTBE produced good recoveries for both mitragynine and ajmalicine. However, the use of ethyl ether can result to a larger deviation for the mitragynine recovery during the extraction process, due to this solvent's high volatility; therefore, MTBE was selected as the solvent for extraction of mitragynine through the remaining method development.

3.4. Quality control and method validation

A performance assay was carried out on urine samples spiked with mitragynine at seven concentrations ranging from 0.01 to 5 ng/ml, and processed as described in Section 2.6. All spike levels were run in triplicate. The linear regression plot of the area ratios (analyte/IS) versus the compound's concentration ratios with a weighting factor of 1/*x* (*x*: concentration) indicated an accuracy of 90–115%, and a correlation factor $r > 0.995$.

Three QC samples at 0.1, 1, and 5 ng/ml, representing low, middle and high spiking levels, were used for method validation; this concentration range is suited both to the instrument sensitivity, and to the liquid–liquid extraction of mitragynine in human urine. If a sample contains mitragynine at a concentration above the calibrated linear range, then an appropriate dilution of extract is required.

Matrix effects were studied by comparing the retention times and peak areas of mitragynine in neat solutions, pre-extraction spiked and post-extraction spiked urinary extracts in concentrations from 0.025 to 2.5 ng/ml. The retention times were not

Table 2
Mean extraction recoveries of mitragynine (analyte) and ajmalicine (IS) at level of 1 ng/ml in different solvents (five replicates each).

Solvent	Mitragynine		Ajmalicine	
	Mean recovery, %	RSD	Mean recovery, %	RSD
Ethyl acetate	49	13	60	15
Ethyl ether	82	12	90	10
MTBE	81	8	92	8

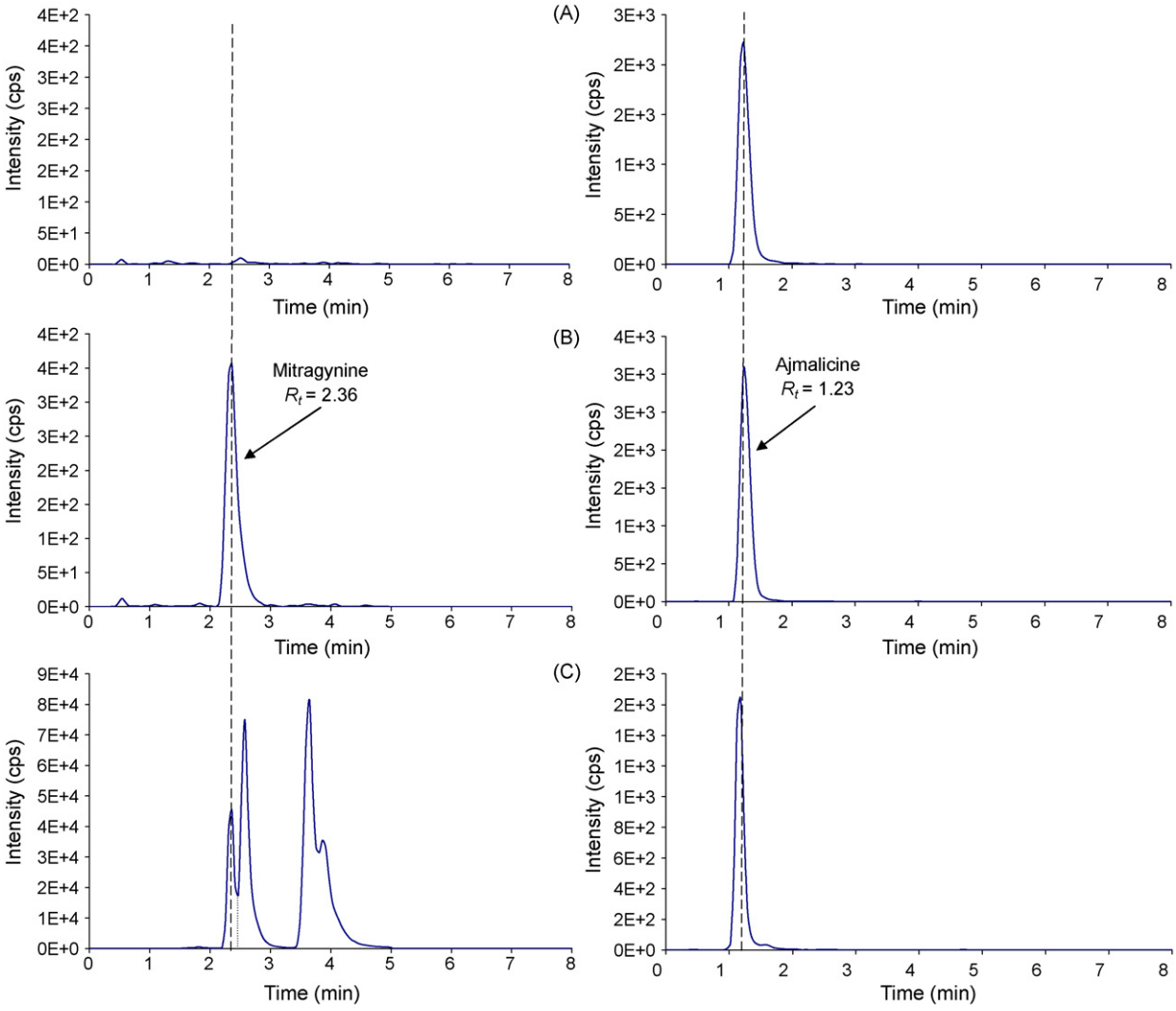

Fig. 5. HPLC-MS/MS extracted chromatograms of mitragynine (left) and ajmalicine (right). (A) Blank urine, (B) 5 ng/ml standard solution, and (C) urine extract from a kratom user. The transitions of *m*/*z* 399 > 174, 399 > 226, and 399 > 238 were used to monitor mitragynine, and the transition of *m*/*z* 353 > 144 was used for ajmalicine.

changed and the mitragynine extraction recovery yields, without IS adjustment, were ranged from 53 to 88%. The peak areas of neat solutions and post-extraction spiked extracts were identical (data not shown). Therefore, there were no significant matrix effects found in our analytical method.

The low system background of mitragynine in the blank urine sample is demonstrated in Fig. 5A. The overall recoveries of mitragynine were found to be between 80 and 110% across the three spiking levels of 0.1, 1, and 5 ng/ml, and for both intra-day and inter-day studies. Intra-day assay precision (RSD) values were respectively 12 and 16% for matrix spiked at 1 and 5 ng/ml urine. The lowest spiking level, at 0.1 ng/ml, yielded a higher RSD, 22% (Table 3). The accuracy of the method is in agreement between the spiking concentrations and the measured values.

Table 3
Intra-day assay precision for mitragynine determination in human urine in triplicate for each level.

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	RSD
0.1	0.1	22
1.0	1.1	12
5.0	4.9	16

The maxima of inter-day assay precision of RSD 33, 16 and 16% were obtained at concentrations of 0.1, 1.0 and 5.0 ng/ml urine, respectively (Table 4). No significant differences in mitragynine recoveries were observed in the intra-day and inter-day studies. Both results are within the range of acceptance criteria.

The MDL, determined in seven replicates, for mitragynine in urine was 0.02 ng/ml (RSD = 21%). The LLOQ of mitragynine in urine was 0.1 ng/ml. The MDL and LLOQ for this method are much lower than the values reported previously by Janchawee et al. [16] using an HPLC-UV detection.

Table 4
Inter-day assay precision for mitragynine in urine measured in triplicate for each level.

Analysis time (age of sample)	0.1 ng/ml		1 ng/ml		5 ng/ml	
	Mean recovery, %	RSD	Mean recovery, %	RSD	Mean recovery, %	RSD
Fresh	90	22	109	12	98	16
1 day	80	33	93	16	94	5
7 days	90	11	102	7	96	10
28 days	110	9	115	13	103	8

3.5. Application

A forensic sample from an alleged kratom user was provided by the Upstate New York Poison Control Center, SUNY Upstate Medical University, and was kept at −80 °C until analysis. The preliminary analysis of the urine extract showed that mitragynine level was largely above the calibration range. The sample was then diluted 20-fold with methanol and analyzed in triplicate to enable a precision determination at a 95% confidence level. An amount of 167 ± 15 ng/ml mitragynine residue was found in the sample as evident from the HPLC-MS/MS (Fig. 5C). The recorded MS/MS transitions of m/z 399 > 174, 399 > 226, and 339 > 238, which are characteristics of mitragynine molecule, confirmed the presence of this compound in the urine extract.

4. Conclusion

The accessibility of kratom as a form of illicit substance abuse has increased via the Internet. Until now, there has been no means to detect or confirm drug exposure thus making any and all clinical observations related to suspected use entirely speculative. Consumption of kratom can lead to a detectable content of mitragynine residue and its metabolite in urine. Mitragynine residue in urine sample was extracted using MTBE and analyzed on HILIC column coupled to a tandem mass spectrometry. Ajmalicine was found to be a suitable IS both for the extraction and the HPLC-MS/MS analysis of mitragynine. High accuracy, precision, and sensitivity were demonstrated for HPLC-MS/MS analysis of mitragynine in urine matrix, with detection and quantitation limits of 0.02 and 0.1 ng/ml, respectively. The availability of a certified mitragynine standard would facilitate analysis and monitoring studies.

The development of this method will make further clinical observation and future research possible relative to the acutely poisoned patient. The study of mitragynine metabolites and solid phase extraction (SPE) will be developed in a near future.

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