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Technical Note

Screening and Identification of Mitragynine and 7-Hydroxymitragynine in Human Urine by LC-MS/MS

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Abstract: Kratom is a tree planted in Southeast Asia, including Thailand, Malaysia, Myanmar (Burma) and elsewhere in the region. A long history of usage and abuse of kratom has led to the classification of kratom as a controlled substance in its native Thailand and other Southeast Asian countries. However, kratom is not controlled in the United States, and the wide availability of kratom on the Internet and in the streets has led to its emergence as an herbal drug of misuse. With the increasing popularity of kratom, efficient protocols are needed to detect kratom use. In this study, a rapid method for the analysis of kratom compounds, mitragynine and 7-hydroxymitragynine, in human urine has been developed and validated using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The chromatographic system employed a 2.6- μ m 100 mm \times 2.1 mm phenyl-hexyl analytical column and gradient elution with a 0.4-mL/min flow rate of water and acetonitrile as mobile phases. A triple quadrupole mass spectrometer was used as the detector for data acquisition. The analyst was the quantification software. The established method demonstrated linearity of >0.99 for both analytes, and low detection limits were obtained down to 0.002581 ng/mL for mitragynine and 0.06910 ng/mL for 7-hydroxymitragynine. The validated method has been utilized for clinical analysis of urine for the purpose of mitragynine and 7-hydroxymitragynine detection.

Keywords: mitragynine; 7-hydroxymitragynine; kratom; LC-MS/MS

1. Introduction

Kratom has long been used by opioid-dependent individuals as an alternative to their unavailable opioid of choice and chronic pain medication, as a stealth-to-urine drug screening opiate substitute while in opioid recovery treatment and recreationally, alone or as a booster [1–3]. However, it has also been used to reduce opioid craving [4,5] and to treat opioid withdrawal [6,7]. Kratom is derived from the Mitragyna speciose Korth tree in Southeast Asia, and like oxycodone, it has rare paradoxical properties consisting of augmented focus, concentration and physical energy, with concurrent reduction in physical and emotional pain [8,9]. Kratom was federally outlawed in Thailand by 1950 and in 2003 by Malaysia [10,11]. The U.S. Drug Enforcement Administration (USDEA) Office of Diversion Control lists kratom as a drug of concern; however, kratom remains legal in the U.S. and is also one of the most popular legal highs in the U.K. [12–15]. M. speciose Korth contains more than 25 alkaloids that vary quantitatively depending on geographic location [5,10,16–18].

Mitragynine (Figure 1) is structurally similar to the aphrodisiac yohimbine, which is the most prevalent of these alkaloids, and mitragynine is believed to be responsible for kratom's opioid effects [19]. Although mitragynine is structurally distinct from morphine and codeine, it acts on a supraspinal mu- and delta-opioid receptor and serotonergic and noradrenergic pathways in the spinal cord [20]. Mitragynine is considerably more potent than morphine [10,21,22] and is thought to act centrally on mu-opioid receptors within the nucleus accumbens to produce its addictive cycle of anhedonia followed by hedonia followed by anhedonia [23–27], the definition of progression, tolerance and addiction. Kratom products are available as leaves, gum and powder, and the alkaloid is usually brewed into tea or smoked [28–30]. The effects of kratom depend on the dose used, beginning within 5 to 10 min and lasting for about 1 h [31–34]. The kratom high varies and is dose dependent. Lower doses tend to yield a dopaminergic-dominant result or stimulation, while higher doses result in a mu-opioid receptor-dominant effect, which eclipses the dopaminergic expression or physical and emotional hedonia and pain relief [12,24,35,36].



Mitragynine (MW 398.50)7-Hydroxymitragynine (MW 414.49)C23H30N2O4C23H30N2O5

Figure 1. Chemical structure of "kratom" compounds.

Many analytical approaches have been reported for the separation and identification of kratom, including GC and GC-MS [2,3,37–39], LC and LC-MS [38,40–44] and CE and CE-MS [43,45,46]. In this study, we report a rapid and effective method for quantification mitragynine and 7-hydroxymitragynine compounds in human urine matrix. Correlation coefficients greater than 0.99 were obtained for both analytes, and limits of detection down to 0.002581 ng/mL and 0.06910 ng/mL were achieved for mitragynine and 7-hydroxymitragynine, respectively.

2. Experimental Section

2.1. Chemicals and Materials

Acetonitrile and HPLC-grade water are purchased from EMD Millipore (Billerica, MA, USA). Formic acid was purchased from Amresco (Solon, OH, USA). Mitragynine (Item Number M-152, Lot Number FN102312-03) and 7-hydroxymitragynine (Item Number H-099, Lot Number FN122812-01) standards were purchased from Cerilliant (Round Rock, TX, USA). Internal standards mitragynine-D3 (Item Number M-182, Lot Number FN06021404) and 7-hydroxymitragynine-D3 (Item Number H-109, Lot Number FN05291405) were purchased from Cerilliant (Round Rock, TX, USA). The phenyl-hexyl HPLC column was purchased from Phenomenex (Torrance, CA, USA).

2.2. Instrumentation

The assay was developed on a Shimadzu 20AD liquid chromatography (Columbia, MD, USA) coupled to an AB Sciex QTrap 5500 quadrupole linear ion trap mass spectrometer (Framingham, MA, USA). A 2.6- μ m 100 mm × 2.1 mm phenyl-hexyl analytical column was employed, and gradient elution with a 0.4-mL/min flow rate of water and acetonitrile as mobile phases was utilized. The LC-MS/MS conditions were optimized to achieve rapid and effective goals for the detection of kratom compounds.

2.3. Standard Solutions

Calibration standard solutions were prepared weekly to keep the active component fresh. Methanol standards and urine standards were prepared for injection, respectively. The methanol standards were stored at -8 °C, and the urine standards were stored at 4 °C. For HPLC injection, 50 µL of working standards, 50 µL of 10 ng/mL internal standards and 150 µL of Mobile Phase A solution were mixed as the injection standards. For testing on urine samples, working standards were substituted with human urine.

3. Results and Discussion

3.1. HPLC Conditions

A phenyl-hexyl HPLC column was employed to develop the assay, as previous studies showed that the column had a wide range for pH suitability. In addition, the phenyl-hexyl column provides increased aromatic selectivity and increased hydrophobic retention. The phenyl phases tend to exhibit good shape selectivity, and with a controlled, low-level surface charge inherent to the particles in combination with a tri-functionally-bonded phenyl-hexyl ligand, the phenyl-hexyl column provides an exceptional peak shape for basic compounds, even in the ionized form under acidic mobile phase conditions. The column was maintained in an oven with 40 °C. Analytes were eluted with gradient mobile phases of water with 0.1% formic acid (Mobile Phase A (MPA)) and acetonitrile (Mobile Phase B (MPB)). Formic acid is a commonly-used additive for reversed-phase liquid chromatography, as it provides protons and promotes ionization for analytes. Acetonitrile is an organic solvent that provides advantages over methanol in terms of low back pressure, high sensitivity and less ghost peak for the gradient elution program.

The gradient elution starts with 5% MPB, with a linear gradient to 75% within 3 min, followed by a 3-min stabilization. Then, the percentage of MPB drops to 20% for post-run equilibrium. The optimized separation results are presented as Figure 2.



Figure 2. The extracted ion chromatogram of kratom. The major components of kratom, mitragynine and its major metabolite, 7-OH-mitragynine, are shown. Peak identification: (1) 7-hydroxymitragynine; (2) mitragynine. Concentration: 5 ng/mL for both compounds.

3.2. Mass Spectrometry Parameters

Before connecting to LC, the direct infusion experiments were carried out for exploring optimum mass spectrometry conditions. Different parameters, such as ion spray voltage, ionization temperature, were explored. The optimum mass spectrometer conditions are demonstrated as Table 1.

Ion Spray Voltage	2500 V	
Temperature	600 °C	
Collision Gas	Medium	
Ion Source Gas 1	50.0	
Ion Source Gas 2	70.0	

Table 1. Mass spectrometer conditions.

After mass spectrometer conditions finalized, multiple reaction monitoring (MRM) parameters were determined. For drug analytes, two transitions were tracked. For the internal standards, one transition was monitored. The values are presented in Table 2.

Table 2. MRM parameters for analytes and internal standards.

Compound	Precursor ion (m/z)	Product ion (m/z)	Collision energy (volts)
Mitragynine	399.3	174.1/159.0	40/55
Mitragynine-D3	402.3	238.1	30
7-Hydroxymitragynine	415.3	190.0/238.1	40/27
7-Hydroxymitragynine-D3	418.3	193.1	40

3.3. Method Validation

Once the assay was developed, we proceeded to the validation steps. Validation is a vital part before an assay can be applied to routine testing. Several parameters were studied as the parts of the validation process.

3.3.1. Linearity

Six concentration levels (2, 3.75, 5, 15, 25 and 50 ng/mL) with six replicates of each level were used for establishing the regression model.

For mitragynine, the accuracy of the concentrations of analytes at all levels is within $\pm 10\%$ of the expected value, except at the lower limit of quantification (0.08 ng/mL), where concentrations are within $\pm 15\%$ of the expected values. These data qualify for the requirement of the validation plan, which asks for an accuracy within $100\% \pm 25\%$. The percent of the relative standard deviation (%RSD, or percent of the coefficient of variation, %CV) is below 10% at all concentration levels, which qualifies for the requirement of the validation plan (less than 25% CV). The coefficient of determination (R²) is 0.9941 for mitragynine. This demonstrates that the calibration model of mitragynine is close to linear (=1) and is valid for quantification.

For 7-hydroxymitragynine, the accuracy of the concentrations of analytes at all levels is within $\pm 10\%$ of the expected value. These data qualify the requirement of the validation plan, which asks for accuracy within $100\% \pm 25\%$. The percent of the relative standard deviation (%RSD, or percent of the coefficient of variation, %CV) is below 10% at all concentration levels, which qualifies the requirement of the validation plan (less than 25% CV). The coefficient of determination (R²) is 0.9951 for 7-hydroxymitragynine. This demonstrates that the calibration model of 7-hydroxymitragynine is close to linear (=1) and is valid for quantification.

3.3.2. Carryover

Carryover is measured by evaluating blank urine samples injected after the highest concentration samples in the calibration curve.

For mitragynine and 7-hydroxymitragynine, the back-calculated concentration of the blank sample was "0 ng/mL", which means the carryover condition of the system is acceptable.

3.3.3. Sensitivity

Sensitivity is measured as the limit of detection (LOD) and the limit of quantification (LOQ). Four concentration levels, 0.01, 0.02, 0.04 and 0.08 ng/mL, were used to determine LOD and LOQ. Three calibration curves with duplicate injections at four levels were performed.

To calculate LOD and LOQ, the standard deviation of the three y-intercepts (SD_{Yint}) is first calculated along with the mean of the three slopes (means). The LOD equals $(3.3 \times SD_{Yint})/means$, and the LOQ equals $(10 \times SD_{Yint})/means$.

For mitragynine, LOD down to 0.002581 ng/mL and LOD down to 0.007821 ng/mL were obtained. These data demonstrate that the method is very sensitive for both qualification and quantification for mitragynine.

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For 7-hydroxymitragynine, LOD down to 0.06910 ng/mL and LOD down to 0.2094 ng/mL were obtained. These data demonstrate that the method is very sensitive for both qualification and quantification for 7-hydroxymitragynine.

3.3.4. Precision

Precision is measured by analyzing two concentrations (here, low QC and high QC) in duplicates of analytes for total ten batches. It is recommended to analyze two batches per day for five days; or this can be done for one day per batch. However, no more than two batches per day is allowed, and a minimum of 2 h between batches should be assessed. In this way, within-day (intraday) and day-to-day (interday) precision will be evaluated.

Here, precision data from the following batches, ten batches in total, are presented.

For both mitragynine and 7-hydroxymitragynine, %CV at all concentration levels is less than 15%, which meets the requirement (<25%).

3.3.5. Stability

Stability is measured by analyzing two concentrations (here, low QC and high QC) of analytes. We analyze the samples to establish time zero (T_0), then after a certain time span (T_x), samples are re-analyzed. Results will be compared between T_0 and T_x .

3.3.5.1. Short-Term Stability (Autosampler Stability)

Autosampler stability was evaluated for a 92-h time span. This simulates the situation in which samples were prepared on Friday, being processed during the weekend, and then data collected on Monday morning.

For both mitragynine and 7-hydroxymitragynine, T_x concentrations (x = 92 h) at all concentration levels are within ±0.3% of T₀, which meets the requirement (T_x within ±25% of T₀). The data of the short-term stability study are presented as Table 3.

	Mitragynine		7-hydroxymitragynine		
	Low QC	High QC	Low QC	High QC	
T_0	0.1502 ng/mL	1.0000 ng/mL	0.7502 ng/mL	4.9983 ng/mL	
T _x	0.1498 ng/mL	0.9997 ng/mL	0.7502 ng/mL	5.0000 ng/mL	
% Difference	0.266%	0.03%	0%	-0.034%	

Table 3. Data of the short-term stability study for a period of 92 hours (n = 3).

3.3.5.2. Long-Term Stability (Storage Stability)

Storage stability, in Ultimate Analysis Laboratory, refers to specimen storage for two weeks in the fridge after the initial received date. Hence, storage stability was evaluated for a two-week time span.

For both mitragynine and 7-hydroxymitragynine, T_x concentrations (x = 2 weeks) at all concentration levels were within ±0.3% of T₀, which meets the requirement (T_x within ±25% of T₀). The data of the long-term stability study are presented as Table 4.

	Mitragynine		7-hydroxymitragynine		
	Low QC	High QC	Low QC	High QC	
T_0	0.1500 ng/mL	1.0017 ng/mL	0.7500 ng/mL	4.9967 ng/mL	
T_x	0.1503 ng/mL	0.9990 ng/mL	0.7500 ng/mL	5.0000 ng/mL	
% Difference	-0.20%	0.27%	0%	-0.066%	

Table 4. Data of the long-term stability study for a period of 14 days (n = 3).

3.3.6. Matrix Effect Study

The matrix effect is a type of assay interference caused by ion suppression or ion enhancement in the matrix. To test for the matrix effect, five injections of the standards were made, and the results were compared with the "complicated matrix", which in the authors' case, were human urine samples. The % differences between the standards and the urine samples are calculated. The results obtained were 35%, which qualifies for the requirement of the validation plan (40%).

3.4. Capability of the Established Method

After the method was developed and validated, spiked urine samples were utilized to examine the capability of the method. Different concentration levels of standard drugs were spiked into synthetic urine to prepare run solutions (0, 5, 25, 50, 100 ng/mL). The results demonstrated that the recovery efficiency of both analytes was above 95%.

An unknown sample was examined using the established method and the extracted ion chromatogram is shown as Figure 3. The results were 51.25 ng/mL of mitragynine and 8.72 ng/mL of 7-hydroxymitragynine. These numbers exceed our cut-off values, which indicates that the sample was positive for both mitragynine and 7-hydroxymitragynine. Two quality control (QC) standards of 3.5 ng/mL and 25 ng/mL were utilized to monitor the data consistence and instrument performance. Table 5 shows the performance of the QCs.



Figure 3. Extracted Ion Chromatogram of an unknown sample.

Analyte Peak Name (MRM Transition)	Mean Calculated Concentration (ng/mL)	Std. Deviation (ng/mL)	%CV	Number of Values Used	Mean Accuracy (%)
Mitragynine1 (399.300/174.100 Da)	3.594	0.004	0.12	2	95.84
Mitragynine 2 (399.300/159.000 Da)	3.398	0.057	1.69	2	90.60
7-hydroxymitragynine 1 (415.300/190.000 Da)	3.884	0.048	1.24	2	103.57
7-hydroxymitragynine 2 (415.300/238.100 Da)	3.516	0.059	1.69	2	93.75
mitragynine 1 (399.300/174.100 Da)	24.616	0.353	1.44	2	98.46
mitragynine 2 (399.300/159.000 Da)	23.915	0.282	1.18	2	95.66
7-hydroxymitragynine 1 (415.300/190.000 Da)	24.844	0.107	0.43	2	99.37
7-hydroxymitragynine 2 (415.300/238.100 Da)	27.241	0.678	2.49	2	108.96

Table 5. QC summary for daily analysis of mitragynine and 7-hydroxymitragynine (29 Jan 2015).

4. Conclusions

In this study, a specific and selective LC-MS/MS method has been developed for detecting the use of mitragynine and 7-hydroxymitragynine in urine samples. The procedure utilizes a quantitative MRM procedure for the detection of both analytes. The assay has been validated and can be used for routine drug testing of both drugs. Overall, the assay exhibits a rapid and efficient means for the identification of kratom-related compounds in human urine.

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Author Contributions

Hanzhuo Fu: designed and performed the experiment, did the data analysis, manuscript writing and revision. Frank X. Cid: laboratory and instrument support, project design and supervision, as well as manuscript revision. Nat Dworkin: project design and supervision, as well as manuscript revision. James Cocores: manuscript Introduction preparation, critical reading and manuscript revision. Gloria Shore: critical reading and manuscript revision. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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