American Journal of Chemistry 2016, 6(2): 60-64
DOI: 10.5923/j.chemistry.20160602.06

Qualification and Quantitation of Kratom Compounds in Human Urine by High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract Mitragynine and its major metabolite, 7-hydroxymitragynine (also known as "Kratom"), are drugs which extracts from a tree planted in Thailand, Myanmar and other Southeast Asian countries. Nowadays, kratom compounds are used as recreational drugs in many countries all over the world. Chromatography coupling to mass spectrometry becomes a vital instrumentation tool for the separation and determination of small molecules qualitatively and quantitatively. The previous report studied a novel method for screening and identification of mitragynine and 7-hydroxymitragynine in human urine by liquid chromatography tandem mass spectrometry. However, the method conditions were not optimized. The objective of this study is to explore a rapid approach to separate and identify the kratom compounds by new instrumentation-ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) was utilized to perform method development and optimization. Experiments were carried out using a state-of-the-art ultra high performance liquid chromatography system. The method was found linear in the range of 10 - 100 ng/mL. In the linearity study, good regression equation and correlation coefficient were achieved. Comparing to HPLC system, UHPLC exhibited short analysis time and high selectivity. The optimized method can be used for the separation and detection of mitragynine and 7-hydroxymitragynine.

Keywords Mitragynine, 7-hydroxymitragynine, Liquid chromatography, Mass spectrometry

1. Introduction

Mitragynine and its major metabolite, 7-hydroxymitragynine (also known as "Kratom"), are drugs which extracts from a tree planted in Southeast Asian countries [1]. Nowadays, kratom compounds are used as recreational drugs in many countries all over the world. Mitragynine and 7-hydroxymitragynine (physical properties shown as Table 1) is structurally similar to each other [2, 3].

A number of analytical methodologies are available for qualitative and quantative determination of drugs of abuse and metabolites. For instance, gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) are widely utilized as a powerful tool in the toxicology applications due to its high selectivity and relative low maintenance cost. Meyer et. al. studied the metabolism of the designer drugs of mephedrone, butylone, and methylone in urine using gas chromatography–mass spectrometry [4]. Bergot *et. al.* developed a method for quantitative determination of the four known juvenile hormones in insect tissue using gas chromatography—mass spectroscopy [5]. Capillary electrophoresis is another powerful tool which is widely used due to its high efficiency and advantages for charged and neutral compounds analysis. Merola *et. al.* developed a method for chiral separation of 12 cathinone analogs by cyclodextrin assisted capillary electrophoresis with UV and mass spectrometry detection [6]. Capillary electrophoresis is also used in different applications such as environmental, food and agricultural sciences. Woolley *et. al.* developed ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips [7]. Zheng *et. al.* reported a rapid method for separation and identification of microcystins using capillary electrophoresis and time-of-flight mass spectrometry [8].

Chromatography is a separation technique which is widely used for various applications, including petroleum products, fast consumption products, drug analysis, amino acids, protein purifications and extractions, and so on.

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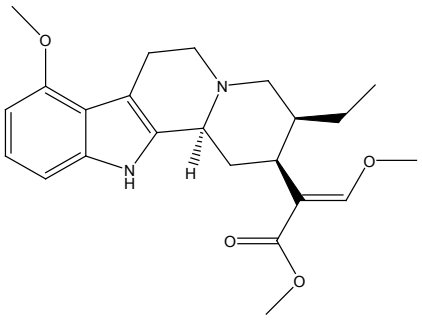
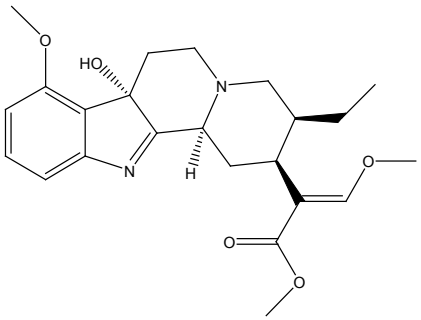
Published online at http://journal.sapub.org/chemistry

Table 1. Physical properties of mitragynine and 7-hydroxymitragynine

Parameters	Mitragynine	7-Hydroxymitragynine
Chemical formula	$C_{23}H_{30}N_2O_4$	$C_{23}H_{30}N_2O_5$
Molecular weight	398.50	414.49
Chemical structure		
IUPAC name	(E)-2-[(2S,3S)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[3,2-h]quinolizin-2-yl]-3- methoxyprop-2-enoic acid methyl ester	(αE,2S,3S,7aS,12bS)-3-Ethyl-1,2,3,4,6,7,7a,12b-octahydro-7a-hydroxy-8-methoxy-α-(methoxymethylene)indolo[2,3-a]quinolizine-2-acetic acid methyl ester
CAS Registry Number	6202-22-8	174418-82-7

As a new trend, chromatography coupling to mass spectrometry becomes a vital instrumentation tool for the separation and determination of various compounds in different applications. For instance, Zhao *et. al.* utilized liquid chromatography coupling to quadrupole time-of-flight mass spectrometry (Q-TOF) to study the reductive and oxidative degradation of iopamidol, iodinated X-ray contrast media, by Fe(III)-oxalate under UV and visible light treatment [9]. Chen *et. al.* studied TiO_2 photocatalytic degradation and detoxification of cylindrospermopsin by state-of-the-art ion trap mass spectrometry technology [10] and Liu et. al. reported the analysis of microcystis aeruginosa growth and associated microcystin cyanotoxin remediation with similar techniques [11]. Baygildiev *et. al.* presented hydrophilic interaction liquid chromatography–tandem mass spectrometry methylphosponic and alkyl methylphosphonic acids determination in environmental samples after pre-column derivatization with p-bromophenacyl bromide [12]. Scholz et. al. reported heating two types of enriched margarine: complementary analysis of phytosteryl/ phytostanyl fatty acid esters and phytosterol/phytostanol oxidation products [13]. Zhao et. al. studied photocatalysis of 6-hydroxymethyl uracil, a model compound for the potent cyanotoxin cylindrospermopsin using liquid chromatography with UV detection [14].

A number of scientific methods have been studied for the determination of kratom, including gas chromatography [15, 16], liquid chromatography [3, 17-20], capillary electrophoresis [21], and different techniques coupling to mass spectrometry and other technique methods. Fu *et al.* reported a novel method for screening and identification of mitragynine and 7-hydroxymitragynine in human urine by liquid chromatography tandem mass spectrometry [3]. The separation was done within 5 min and the product ions were detected by tandem mass spectrometry. However, the method conditions still have rooms to be optimized. The objective of this study is to explore a rapid approach to separate and identify the kratom compounds by new instrumentation-ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

2. Experimental

2.1. Reagents

Acetonitrile and LC-MS grade water were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). Mitragynine and 7-hydroxymitragynine standards were purchased from Cerilliant Chemicals (Texas, USA). The C-18 column was purchased from Agilent Technologies. Calibration standard solutions were prepared prior to method development and stored at -4°C to keep the active component effective.

As presented in Table 1, mitragynine and 7-hydroxymitragynine have the molecular weight of 398.50 and 414.49, respectively. The chemical formula of mitragynine is $C_{23}H_{30}N_2O_4$ with Chemical Abstract Service (CAS) registry number 6202-22-8, and the chemical formula of 7-hydroxymitragynine is $C_{23}H_{30}N_2O_5$ with CAS registry number 174418-82-7. The difference between the two compounds is only one oxygen in terms of atoms.

2.2. Instrument

The experiments were carried on an Agilent 1290 Infinity LC Systems coupling to a Sciex API 4000 triple quadrupole mass spectrometer. A 2.6-μm 50 mm × 2.1 mm C-18 analytical column was utilized with a 0.6-mL/min flow rate

of mobile phases.

The mass spectrometer was optimized to achieve rapid and effective goals for the objectives using direct infusion techniques before exploring UHPLC parameters. The mass spectrometer parameters are shown as Table 2.

In addition to the above mentioned parameters and the parameters listed in Table 2, a number of other parameters, such as the column length, column diameters, column types, the LC oven temperature, the LC mobile phases, the mobile phase flow rate, gradient program etc. were examined as well. The optimized outcome was illustrated as Fig. 1 extracted ion chromatogram of mitragynine and 7-hydroxymitragynine.

Table 2. Parameters of mass spectrometry determination

Parameter	Mitragynine	7-Hydroxymitragynine
Source	ESI	ESI
Mode	Positive	Positive
Precursor Ion	399.2	415.2
Product Ion	159.1	190.2
Ion Spray Voltage	3500 V	3500 V
Temperature	550 °C	550 °C
Curtain Gas	30	30
CAD	4	4
DP	33	40
EP	11	11
CE	60	55
CXP	9	12

2.3. Preparation of Standard Solutions

Stock solutions of 100 µg/ml of mitragynine and 100 µg/ml of 7-hydroxymitragynine were prepared by dissolution of standard compounds in 100% methanol for long term storage. The stock solutions were further diluted with methanol to prepare working standard solutions at lower concentrations.

3. Results and Discussion

3.1. Method Optimization

Kratom compounds were eluted with mobile phases of water with 0.01% formic acid as mobile phase A) and acetonitrile as mobile phase B). Fig. 1 illustrates the optimized separation results of the two analytes, mitragynine and 7-hydroxymitragynine. The gradient begins with 10% of mobile phase A, with a linear gradient to 60% within 3 min. A 3-minute post run was employed after both compounds eluted.

As demonstrated in Fig. 1, the 7-hydroxymitragynine was eluted first at 1.26 min. The mitragynine was then eluted at 1.38 min. The two compounds were separated under the previously describe conditions.

This method provides a rapid approach for qualitative and quantitative analysis of the mitragynine and 7-hydroxymitragynine, where both compounds were eluted within 2 min.

3.2. Method Examination

The calibration model or linearity of the method is a mathematical representation of the correlation between instrument signal and analyte concentration [3]. To examine the linearity of the developed method, five levels of concentration were used. The peak area ratios (PAR) from these calibrators are then plotted versus concentration. After the data are plotted, they should be fitted (regressed) with a best fit line. While the R^2 or correlation value (r) may be evaluated, it is more appropriate to evaluate residuals. In general, the r should be greater than 0.98 and the residuals (back calculated values) should be within 25% of the expected values. Fig. 2 demonstrates the linearity of mitragynine.

The linearity proves that the conditions of the method were optimized on the instrumentation system within the detection range. Generally, a 0.99 of coefficient of determination represents that a good linear range was achieved in the reported range. As a result, the method can be further used for both mitragynine and 7-hydroxymitragynine compounds in real sample applications.

As illustrated in Fig. 2, five standards at different concentrations were utilized to example the linearity of the established method. The concentration range from 10 ng/mL, 25 ng/mL, 50 ng/mL, 75 ng/mL, and 100 ng/mL were used in the linearity test. Three replicates were used to avoid random errors and the average intensity of the instrument response was utilized for the final calculations.

Table 3 demonstrates the linearity and sensitivity data for CE-MS results. Correlation coefficients, LODs and LOQs are calculated for all analytes, with ranges of 1.0 to 11 ng/mLfor LOD, and 3 to 33 ng/mL for LOQ.

Table 3. Figures of merit for LC-MS/MS results

Analytes	Linearity		Sensitivity		Precision (Intraday, n=3)	Precision (Interday, n=3)
	Slope	R^2	LOD (ng/mL)	LOQ (ng/mL)	RSD%	RSD%
Mitragynine	77.0	0.9932	5.6	16.0	1.5	2.1
7-hydromitragynine	86.2	0.9956	6.1	18.2	1.1	1.7

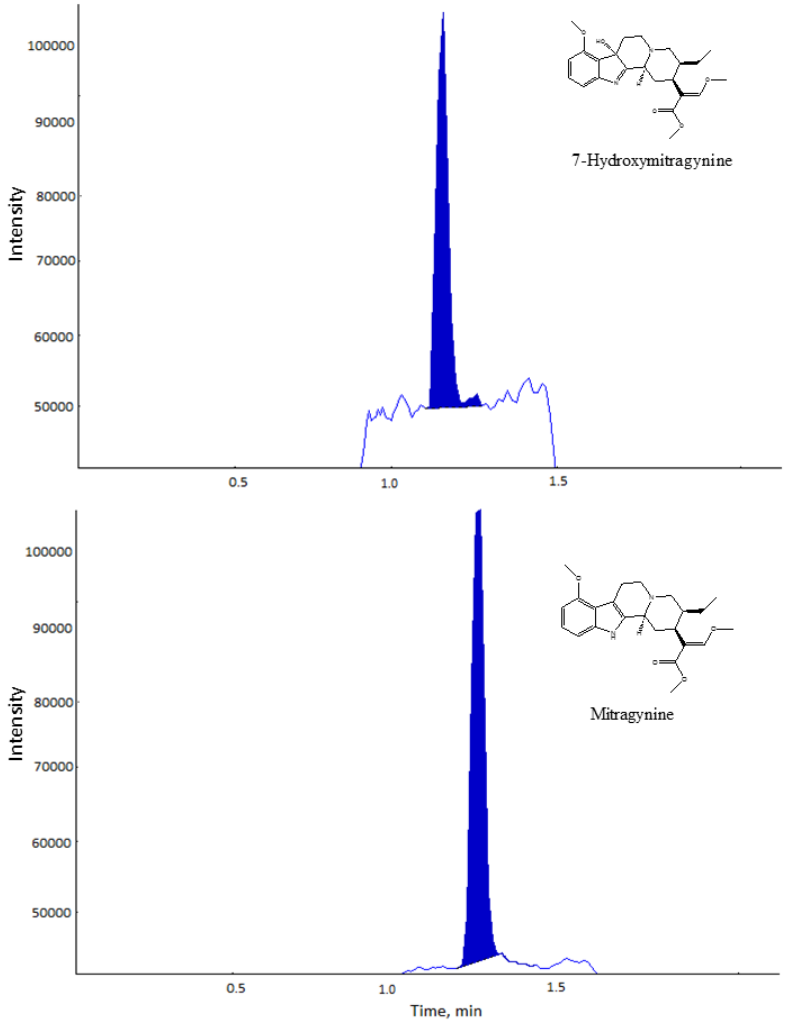


Figure 1. Extracted Ion Chromatogram of mitragynine and 7-hydroxymitragynine

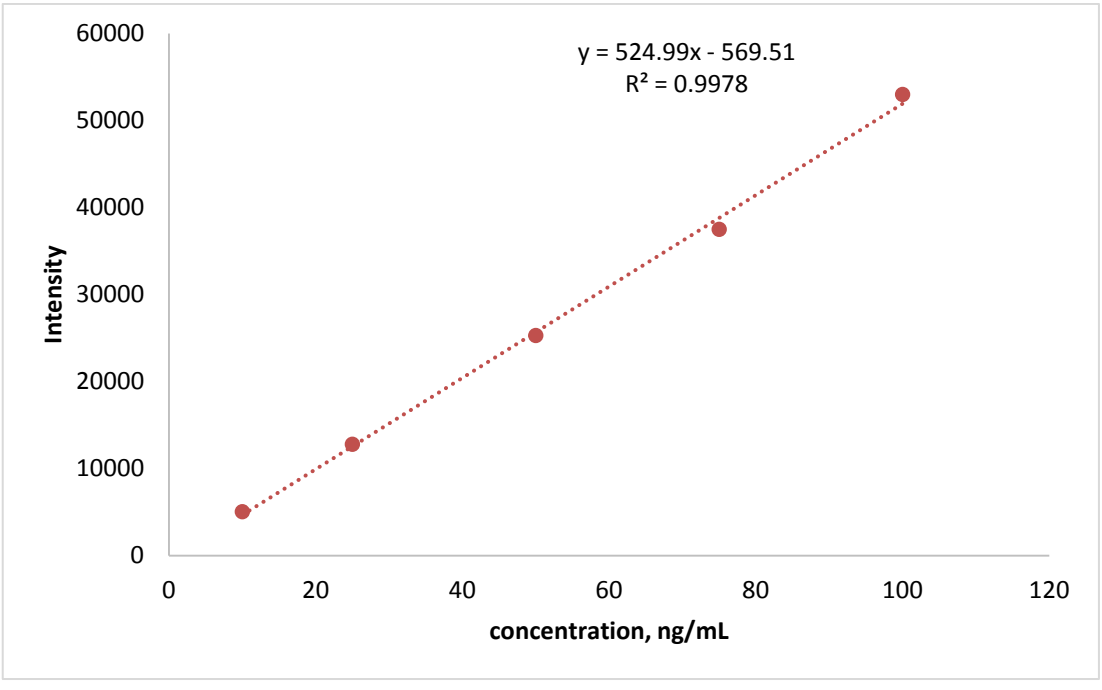


Figure 2. Linearity of mitragynine

3.3. Liquid-liquid Extraction Evaluation

Liquid-liquid extraction is widely used for sample clean-up and sample pre-concentration purposes. The liquid-liquid extractions of mitragynine and 7-hydroxymitragynine were evaluated with two solvents, ethyl ether and ethyl acetate. The ratio between quantified values and control spiked values, termed as the recoveries, were examined using urine samples spiking with a known amount of mitragynine and 7-hydroxymitragynine standards.

The calculation results of mitragynine and 7-hydroxymitragynine using different solvents were presented in Table 4. It can be concluded that both solvents are good choice for liquid-liquid extraction of mitragynine and 7-hydroxymitragynine compounds.

Table 4. Recoveries of liquid-liquid extraction of mitragynine and 7-hydroxymitragynine with different solvents

Solvent	Mitragynine (n=3)		7-hydroxymitragynine (n=3)	
	Recovery (%)	RSD	Recovery (%)	RSD
Ethyl ether	76	15	75	12
Ethyl acetate	51	12	49	10

4. Conclusions

The current study presents a rapid and selective method for the separation and detection of mitragynine and 7-hydroxymitragynine using ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). This method can be applied for routine clinical and forensic applications.

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