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The Validation of Mitragynine And 7-Hydroxymitragynine Determination in Postmortem Blood by Chloroform Extraction

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ABSTRACT

Kratom (Mitragyna speciosa) is traditionally used for medicinal purposes and acceptance as a medicinal plant with recently legal changes. Therefore, Kratom abuse has emerged as a significant public health concern in Thailand, particularly there is linked to traffic accidents. Mitragynine, and 7-hydroxymitragynine are effectively measured in human samples by using LC-MS/MS. The aims of this study were conducted on validations of chloroform use instead of methanol on blood mitragynine, and 7-hydroxymitragynine extraction prior analyzed by on LC-MS/MS. The simulated postmortem blood was the pooling of ten expired blood units and added with different concentrations of mitagynine and 7-hydroxymitragynine (1.0-100 ppb), were extracted by chloroform and analyzed by LC-MS/MS. The results showed that the running time was about 10 min and the retention time (RT) of 7-hydroxymitragynine and mitragynine were 3.631 and 4.703 min, and each m/z profiles were correlated. The linearity of standard curves for mitragynine and 7-hydroxymitragynine were preferable. The LOD and LOQ of both metabolites were within 1.0 ppb as lowest point of standard concentration, whereas the standard concentrations at 10.0, 50.0 and 80.0 ppb were high linearity and preferable for the analytical range. The %CV of precision tests included within a day and between five days at all concentrations were acceptable by within ±20%. The peak area of mitragynine and 7-hydroxymitragynine within postmortem blood (n = 4) by different extractions, were statistically different (p = 0.012), which were implied that the yield of mitragynine and 7-hydroxymitragynine within postmortem blood extracted by chloroform was higher than by methanol.

Keywords: *Mitragyna speciosa, Kratom, mitragynine, method validation, LC-MS/MS*

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1. INTRODUCTION

Mitragyna speciosa commonly known as “Kratom”, is a native tropical tree originated in South-East Asia including Southern of Thailand. Its leaves contain alkaloids as secondary metabolites, are traditionally used for medicinal purposes and acceptance as a medicinal plant with recently legal changes. Kratom is a significant medicinal plant, which is used for analgesic and stimulant. Since, there has traditional uses and potential health benefits, therefore, the risk of dependence is implicated in Thai society. Health complication from long-term kratom use is non-significantly reported among users and non-users. Although regular users have a higher addiction rate and withdrawal symptoms [1, 2]. Kratom abuse has emerged as a significant public health concern in Thailand, particularly there is linked to traffic accidents. The popular cocktail known as “4 × 100”, a mixture of psychoactive substance, which is combines Kratom with cough syrup and tranquilizers [3, 4, 5]. This mixture is associated with fatalities by severe intoxication and impaired driving accidents. Due to both its

stimulant and depressant effects, motor skills and judgment are impaired and criticized for safe driving

The fatal accidented related Kratom and polydrug cocktail usages have been reported and toxicological analyses in postmortem cases revealed the high levels of mitragynine and its metabolites [3-7]. Mitragynine, the primary alkaloid from Kratom leaves, is metabolized by cytochrome P450 enzymes, particularly CYP3A4, and 7-hydroxymitragynine, a major active metabolite that exhibits a higher affinity for mu-opioid receptors than morphine [8, 9]. Mitragynine and its metabolites are effectively measured in human samples by using advanced analytical techniques. In postmortem cases, mitragynine concentrations in blood is ranged from 1.0–422.0 ng/mL, and 7-hydroxymitragynine is identified in 95% of cases [10]. However, mitragynine in postmortem is significantly redistributed with varying concentrations in different biological fluids [11]. The blood samples for LC-MS/MS analysis of mitragynine and its metabolites are prepared by a systematic approach, which is ensure accurate quantification and identification. Blood samples should be collected in appropriate anticoagulant tubes. The protein precipitation or liquid-liquid extraction of the samples is needed to prepare the biological matrix for isolation of the target analytes. In case of different solvents used for blood preparation, the LC-MS/MS method should be validated for parameters such as linearity, limit of detection (LOD), precision, and accuracy. For instance, LODs for mitragynine and its metabolites can be as low as 0.5-2 ng/mL [10, 11]. Recently, Kratom has been legal status for use and there is led to increase its abuse and the effects of social issues and public health will be broader. At this point, the lack of education on substance uses and the need for effective regulation are primary concerns for the regulation of risk from its consumption i.e., acceptable mitragynine and its metabolites concentration for the drivers. The aims of this study were conducted on validations of chloroform solvent use on blood preparation on LC-MS/MS analysis, instead of methanol extraction as standard protocol [12]. The findings will assure the quantitative methods for control or regulating the Kratom abuse in Thailand.

2. MATERIAL AND METHODS

Postmortem blood collection

The data of postmortem blood (N = 100) were obtained from the routine work on general drug screening, Forensic Toxicology Unit, Department of Forensic Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand during October to November 2024. The postmortem whole blood samples (n = 4) collected from medicolegal cases, transferred to EDTA anticoagulant tubes, and positive for mitragynine and 7-hydroxymitragynine, were included in this study for the accuracy evaluation within positive cases. While the pooled whole blood sample was prepared from ten of expired-blood bags, Group O and negative contained for microbials and xenobiotics, which were obtained from Blood Bank Unit, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. In addition, this pooled whole blood was applied for standard curves and other method validations. The study was reviewed and considered by Suan Sunandha Rajabhat University Ethic Committee (COA. 1-002/2025).

Standard and blood preparations

Standard solutions

The external standards including mitragynine and 7-hydroxymitragynine, and internal standard, mitragynine-D3 were purchased from Merck KGaA, Darmstadt. Germany. The stock of standard solutions of mitragynine and 7-hydroxymitragynine were dissolved with methanol and each concentration was 1.0 mg/L. Each external working standard solution was diluted with methanol, which ranged from 1, 5, 10, 20, 50, 80 and 100 μg/L. While the concentration of mitragynine-D3 was 1.0 μg/L.

Blood sample

The 180 μL of pooled whole blood was added with 20 μL of each concentration of working standard solution, 10 μL of mitragynine-D3 (as internal standard), and 800 μL of chloroform, respectively. The mixture was vortexed for 30 sec and centrifuged at 12,000 rpm for 10 min. After that 600 μL of supernatant from mixture was transferred and evaporated under the stream of nitrogen gas at 40 °C for 25 min. The drying material was reconstituted with 100 μL of acetonitrile, then the sample solution was filtered and transferred to insert vial within amber vial before LC-MS/MS analysis.

For another method of liquid-liquid extraction, we were using methanol instead of chloroform. In case of postmortem blood, we were using 200 μL of EDTA whole blood (without standard solution) instead of expired blood.

Mitragynine and 7-hydroxymitragynine analyses

The LC-MS/MS system was performed by Nexera X2E incorporated with LCMS-8060 (Shimadzu, Japan), and the method was modified according to Scientific Working Group for Forensic of Toxicology (SWGTOX) with the following conditions: SPP-C18 column, 2.1 mm in diameter × 100 mm and particle size = 1.8 μm (Shimadzu, Japan); gradient mobile phase, A = acetonitrile and B = 10 M ammonium formate in distilled water with 0.1% formic acid; flow rate, 0.3 ml/min; injection volume, 5 μl. The retention times of mitragynine and 7-hydroxymitragynine were at 4.703 and 3.631 min, respectively. In addition, retention time of internal standard was at 4.694 min. Standard curves for mitragynine and 7-

hydroxymitragynine were plotted from standard analyst concentration and peak area/height, A/H. The results were represented as concentration of analysts in blood, which were interpreted from standard curves [12].

Method validation and statistical analysis

The sensitivity of test was represented as limit of quantification (LOQ) and limit of detection (LOD). LOD was lowest non-zero calibration curve, while LOQ was calculated from SWGTOX formula. There was dependent on the slope average and SD of Y-intercept [12]. While the specificity was already controlled using authentic external standard within standard curve plotting. The chromatogram and m/z of analyst were also distinguished from internal standard, which was similar structure through each sample run.

The precision of tests was represented as percentage of coefficient variation (%CV). The standard curves for mitragynine and 7-hydroxymitragynine were repeated four-time experiments, and linearity and formulae were calculated. According to the linearity of standard curve, we were selected three levels of standard concentration including upper, middle and lower levels for validation of precision. The %CV within run was ten-time repeated experiments of three levels of standards within day, while %CV between run was five-time repeated experiments between five days. The %CV formulae were explained as follows:

%CV within run = (SD of a single run of samples/mean calculated value of samples) × 100

%CV between run = (SD of grand mean for each conc./ grand mean for each conc.) × 100

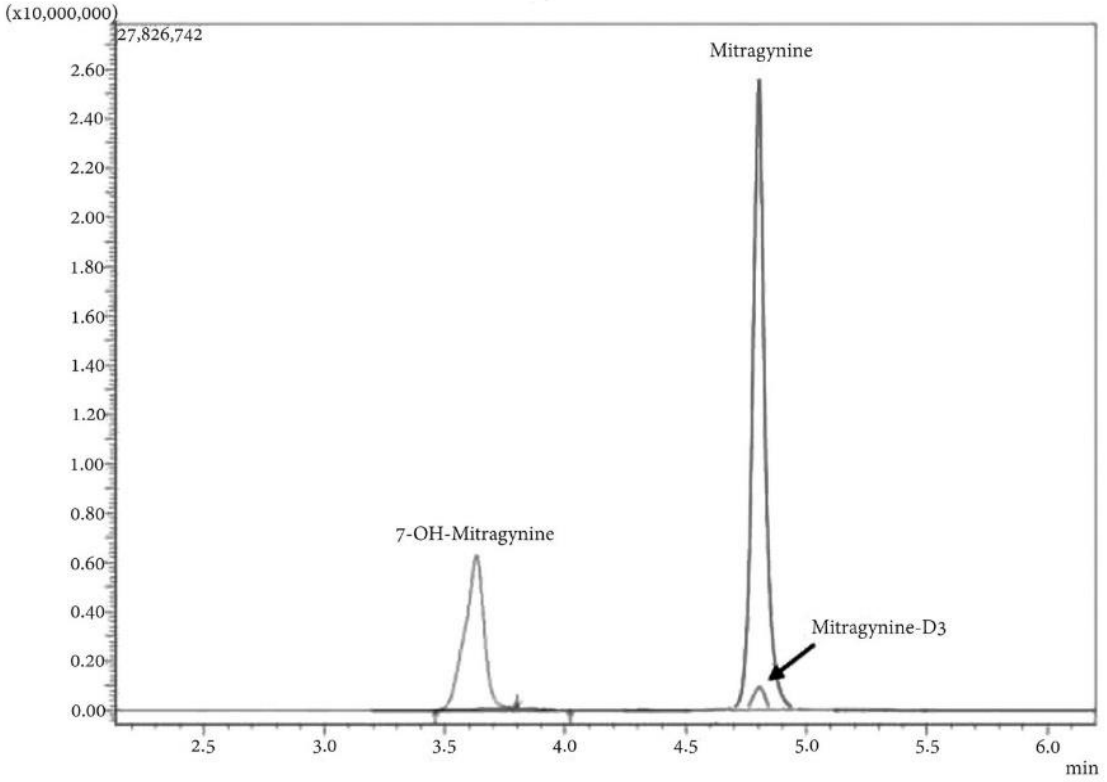
The postmortem whole blood samples from medicolegal cases were positive for mitragynine and 7-hydroxymitragynine, which were used for the accuracy evaluation. The peal area from different blood preparation including routine, methanol and chloroform liquid-liquid extractions were compared.

The differences of linearity between methanol and chloroform blood extractions were represented as formulae and R^2 from different standard curves. LOD, LOQ s and %CV between methanol and chloroform blood extractions were used descriptive statistical analysis. On the accuracy test, differences of peak area among postmortem different blood preparations, including methanol and chloroform extraction, were analyzed by non-parametric rank test, Wilcoxon signed-rank test for statistical analysis.

3. RESULTS AND DISCUSSION

The running time of HPLC was about 10 min and the retention time (RT) of 7-hydroxymitragynine and mitragynine were 3.631 and 4.703 min, while the RT of internal standard, mitragynine-D3 was close to mitragynine, and each m/z profiles were correlated to peak area/height ratio, A/H (Fig. 1). The standard curves of mitragynine and 7-hydroxymitragynine were represented. The linear formulae with r^2 were calculated (Fig. 2), which were strong linearity (r^2 = 0.999). The LOD of mitragynine and 7-hydroxymitragynine were 0.531 and 0.608 ppb, while LOQ was 1.0 ppb for both metabolites (Table 1). Because of their strong linearity, we included standard concentrations at 10, 50, and 80 ppm for precision evaluation.

The %CV of within a day study were 4.43-6.56%, while %CV of between five days were 5.80-10.60%. At the low standard concentration (10 ppm) was higher %CV rather than middle (50 ppm) and upper (80 ppm) levels (Table 2 and 3). Therefore, %CV of both precision tests at all concentration of mitragynine and 7-hydroxymitragynine were acceptable within ±20% and met SWGTOX standard [12]. The peak area of mitragynine and 7-hydroxymitragynine within postmortem blood (n = 4) by different extractions, were tatistically different at p <0.05 (p = 0.012) and there were implied that yield of mitragynine and 7-hydroxymitragynine within postmortem blood extracted by chloroform was higher than by methanol.



Peak	Retention time	m/z	Area	Compound name	A/H
1	3.631	415.1500>190.1000	34555635	7-OH-Mitragynine	5.59
2	4.703	399.0500>173.9500	92488630	Mitragynine	3.615
3	4.694	402.1000>177.0000	2557350	Mitragynine-D3	3.311

Figure 1 The chromatograms of mitragynine and 7-hydroxymitragynine with retention time (min) and mass spectra (m/z)

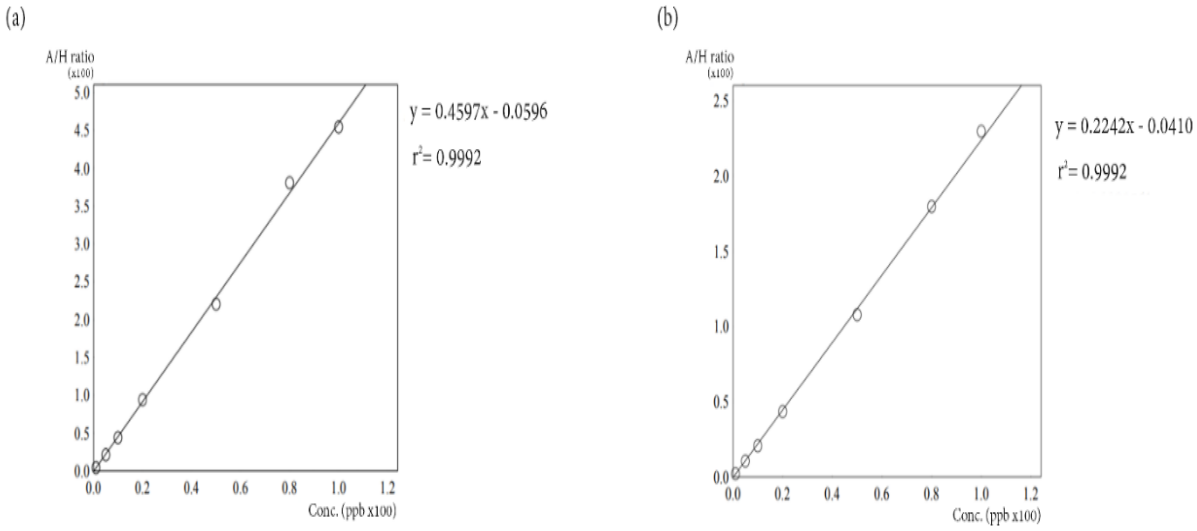


Figure 2 The standard curve (1.0-100.0 ppb) with formula and coefficient of determination (r^2) a) mitragynine; and b) 7-hydroxymitragynine

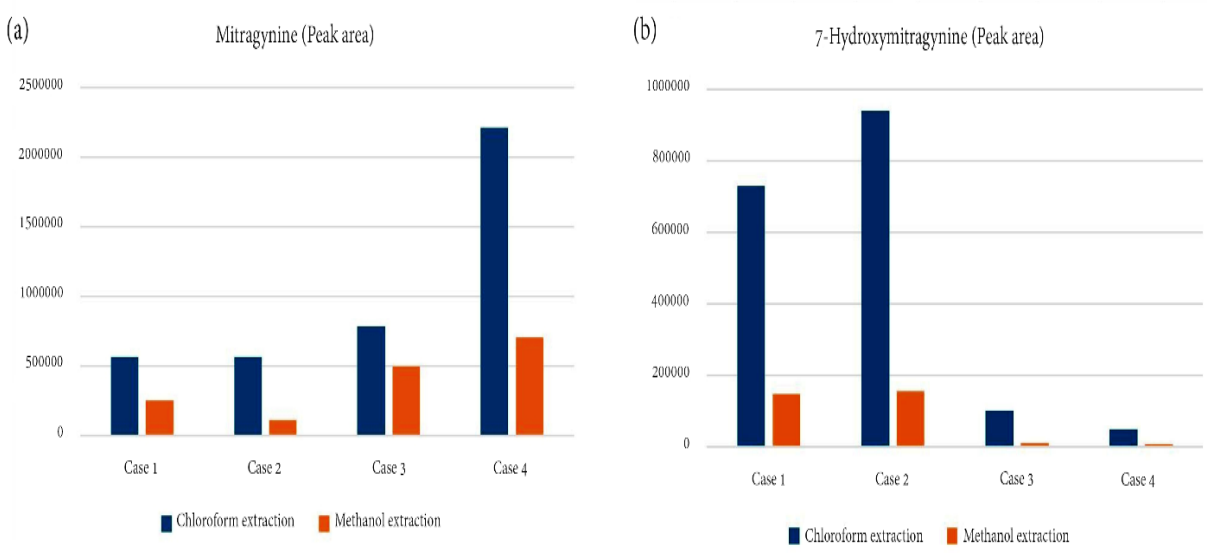


Figure 3 The difference of peak area of a) mitragynine and b) 7-hydroxymitragynine within postmortem blood (n = 4) by different extractions, statistically different at p <0.05 (p = 0.012)

Table 1 LOD and LOQ for the determination of mitragynine and 7-hydroxymitragynine by chloroform extraction

Analyst	**Slope**	**Y-Intercept**	**LOD**	**LOQ**
	(Mean ±SD)	(Mean ±SD)	(ppb)	(ppb)
Mitragynine	0.491 ±0.053	-0.004 ±0.079	0.531	1.0
7-hydroxymitragynine	0.266 ±0.038	-0.040 ±0.049	0.608	1.0

Table 2 Precision within a day for the determination of mitragynine and 7-hydroxymitragynine by chloroform extraction

Analyst	**Precision within a day at different conc.**					
	10 ppb	**(%CV)**	**50 ppb**	**(%CV)**	**80 ppb**	**(%CV)**
Mitragynine	10.0 ±0.65	6.560	50.0 ±6.88	4.990	80.0 ±6.72	4.430
7-hydroxymitragynine	10.0 ±0.46	5.951	50.0 ±4.11	5.174	80.0 ±3.10	5.020

Table 3 Precision between the days for the determination of mitragynine and 7-hydroxymitragynine by chloroform extraction

Analyst	Precision between days at different conc.					
	10 ppb	**(%CV)**	**50 ppb**	**(%CV)**	**80 ppb**	**(%CV)**
Mitragynine	10.6 ±0.49	7.481	56.9 ±2.11	5.802	86.7 ±4.42	6.463
7-hydroxymitragynine	9.5 ±0.29	6.148	54.1 ±2.22	6.195	83.1 ±3.79	6.400

In this chromatographic condition, the highest peak area was mitagynine within pooled-blood samples, which can correlate with postmortem blood that contained mitagynine as major metabolite. In this study, the postmortem blood was simulated by pooling of expired blood units obtained from blood bank and added with different concentrations of mitagynine and 7-hydroxymitragynine, which were free for microbial, and drug contained. In addition, the use of postmortem blood on validation methods was more variation due to drug intake, time and cause of deaths, Thus, the use of the simulated postmortem blood was more reliable. Recently, mitragynine and its active metabolite 7-hydroxymitragynine have been reported that they distribute in various fluid and tissue specimens and comparing central and peripheral blood concentrations in postmortem forensic cases. In addition, the different storage conditions may affect the stability of these compounds [11]. In this study, we had validated mitragynine and 7-hydroxymitragynine analysis by LC-MS/MS was preferable by high sensitivity and specificity according to LOQ and %CV, respectively.

The extraction of mitragynine and its metabolites from postmortem blood involves sophisticated analytical techniques to ensure accurate detection and quantification. Mitragynine, the primary active alkaloid in kratom, poses challenges in forensic toxicology due to its complex chemical nature and the potential for postmortem redistribution. There is known to have minimal postmortem redistribution, which is advantageous for forensic analysis as it reduces the variability in concentration levels between different body sites [11, 13, 14]. Various studies have explored different methodologies for extracting and analyzing mitragynine from biological matrices, each with its own advantages and limitations. LC-MS/MS is widely used for the quantitative analysis of mitragynine in forensic toxicology. It offers high precision and accuracy, making it suitable for complex biological samples. Liquid-liquid extraction (LLE) is another common technique for extracting mitragynine from biological matrices. It involves partitioning the compound between two immiscible liquids, often used in conjunction with high-performance liquid chromatography (HPLC) for detection [15, 16, 17]. Our validation was the use of chloroform instead of methanol on liquid extraction for mitragynine and 7-hydroxymitragynine analysis by LC-MS/MS. Hence, the development of more efficient extraction techniques and advanced analytical tools is crucial for improving the accuracy and reliability of forensic investigations involving mitragynine and its metabolites. Additionally, the legal and regulatory landscape surrounding kratom and its alkaloids remains contentious, influencing the focus and funding of related research [18].

4. CONCLUSION

The validation of blood mitragynine and 7-hydroxymitragynine extraction by chloroform prior LC-MS/MS analysis, instead of methanol extraction. The simulated postmortem blood, pooling of expired blood units and added with different concentrations of mitagynine and 7-hydroxymitragynine, were applied on the validation of blood mitragynine and 7-hydroxymitragynine analyses, which were prepared by chloroform extraction and analyzed by LC-MS/MS. The linearity of standard curves for mitragynine and 7-hydroxymitragynine were preferable. The LOD and LOQ of both metabolites were within 1.0 ppb. The standard concentrations at 10.0 to 80.0 ppb were high linearity and preferable for the analytical range. The %CV of precision tests included within a day and between five days at all concentrations were acceptable by within ±20%. In the postmortem blood, yield of mitragynine and 7-hydroxymitragynine extracted by chloroform was significantly higher than by methanol.

5. CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

6. ACKNOWLEDGMENTS

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