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Udomsak Hoonwijit

Kanok Waithum

Nat Tansrisawad

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Original article

Comparison of mitragynine stability in human blood in common blood collection tubes

Udomsak Hoonwijit*, Kanok Waithum, Nat Tansrisawad

Department of Forensic Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Background: Kratom (*Mitragyna speciosa*) is a plant commonly found in Southeast Asia. Kratom is one of the most abused substances as "a legal psychoactive drug or substance". The main addictive substance of kratom is mitragynine. In forensic toxicology, one of the common problems is that there is no insightful information on mitragynine stability in human blood, so the study of the mitragynine stability would lead to the proper storage of the specimen containing mitragynine.

Objective: To study mitragynine stability in human blood in common blood collection tubes.

Methods: Mitragynine standard was spiked into the blood from the blood bank to get the sample concentration of 500 ng/mL. The sample was divided into 3 groups of common blood collection tubes which were sodium fluoride/potassium oxalate tube, Ethylenediaminetetraacetic acid tube and no anticoagulant tube. The concentrations of mitragynine were then measured and recorded in the conditions after 1 day, 3 days, 5 days, 7 days, 14 days and 30 days.

Results: Comparing the mean concentration of mitragynine with the elapsed time in each tube, it was found that there was no statistical significance ($P > 0.05$) among the blood collection tubes. However, it was found that the mitragynine concentration in sodium fluoride/potassium oxalate tube was more stable than the others. The difference was statistical significance ($P < 0.05$) when the concentrations of mitragynine on day 1 and day 14 were compared in sodium fluoride/potassium oxalate tube while the others revealed the decreasing mitragynine concentration between day 1 and day 7.

Conclusion: This study demonstrated the mitragynine stability in blood in the common blood collection tubes and revealed that the sodium fluoride/potassium oxalate tube was the appropriate collection tube. The limitation of the use of sodium fluoride/potassium oxalate tube to store blood sample for mitragynine determination was about 7 days.

Keywords: Mitragynine, stability in human blood, comparison, blood collection tube.

Nowadays, addictive substances have been regarded as a major problem in most parts of the world. There are several reports of increasing the use narcotics called "Legal High" or "New Psychoactive Substance (NPS)" which consist of stimulants, suppressants and hallucinogens. These drugs or substances are not regulated by laws, therefore, they are currently classified as "Legal psychoactive drugs or substances".[1]

In Europe and North America, one of the legal psychoactive substances is kratom. Since 2010, there has been an increasing rate of kratom abuse. Kratom is recognized as one of the most widely abused plants.[2]

According to Thailand's Narcotic Act 2522 B.E., kratom was classified as Category V. A report of patients who received the treatments from the Ministry of Public Health revealed that kratom is one of the most widely abused substances among drug- addicted people.[3] Currently, kratom has been continuously abused by a variety of methods. Its leaves are harvested for brewing kratom tea. Sometimes, the tea is mixed with other drugs, becoming another mixture of drink, called '*Si Koon Roi'*.

Kratom is a plant, *Mitragyna speciosa*, commonly found in Southeast Asia. Fresh or dried kratom leaves are commonly used by chewing, brewing tea, or mixing with food. If kratom is taken in a low concentration, it would stimulate the nervous

***Correspondence to: Udomsak Hoonwijit**, Department of Forensic Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
E-mail: udomsakhoon@hotmail.com
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system. It could relieve pain of in a moderate level. If it is administered in a high concentration, it will suppress the nervous system.[4] Its two main addictive substances are mitragynine and 7-hydroxymitragynine. The proportion of them is about 60:40.[4] The opioid-like effect of mitragynine is about 13 times higher than morphine.[5] However, there is still limited knowledge of pharmacology and toxicology of mitragynine.

Warmer ML, *et al.* [5] and Feng LY, *et al.* [4] found that kratom was purely used as a single narcotic and mixed with a variety of addictive substances. Lydecker AG, *et al.* [6] reported the concentration of kratom's mitragynine among USA's products as 9.7 - 19 µg/mg in capsule products, and 190.7 - 394.4 ng/µL in beverage products. Holler JM, *et al.* [7] and Neerman MF, *et al.* [8] reported the finding of mitragynine concentrations between 0.23 - 0.39 mg/L with other drugs or substances.

Frequently, forensic doctors discover death due to over abusing addictive substances.[9] However, it cannot be exactly concluded whether mitragynine could only be attributed to the cause of death. Holler JM, *et al.* [7] , Neerman MF, *et al* [8], and Lydecker AG, *et al.* [6] discovered a range of mitragynine concentration between 0.23 and 0.39 mg/L in the dead bodies that also had other types of addictive substances in addition to mitragynine. Due to this fact, the conclusion of death could not only be drawn from only mitragynine intoxication. It was plausible that mitragynine might exist some dangerous reactions between or among other addictive substances.

There is a variety of methods to measure the amount of mitragynine. [10] Mitragynine could simply be determined in a qualitative aspect (e.g. via color test, thin- layer chromatography and gas chromatography). Kowalczuk AP, *et al.* [11] reported the measurement by High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD). Fu H, *et al.* [12] used High Performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) to detect mitragynine in both qualitative and quantitative aspects.

In forensic toxicology, one of the common problems is that there is no complete information of mitragynine stability in human blood. This results in the difficulty of interpreting forensic toxicology results. Consequently, the purpose of this project was to study the stability of mitragynine in human blood in common blood collecting tubes stored in 4 °C refrigerator.

Materials and methods

Ethical approval

This research has been approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University. COA no. 1268/2019 and IRB no. 655/62.

Chemicals and reagent

- Standard: mitragynine, internal standard: mitragynine-D3 in methanol were from Cerilliant® Analytic Reference Standards.
- Acetronitrile (HPLC grade) and formic acid (HPLC grade) from Merk®, 10 mM ammonium formate (Crystalline/Certified ACS) from Fisher chemical®, and QuEChERS : UCT® consisting of Mg_2SO_4 4,000 mg and NaCl 1,000 mg was purchased from Chemical Express Company Limited.

LC-MS/MS conditions

The LC-MS/MS: Shimadzu® 8060 Triple Quadrupole Mass Spectrometer.
- Mobile Phase A: 10 mM ammonium formate and 0.1% formic acid buffer
- Mobile Phase B: 10 mM ammonium formate in methanol + 0.1% formic acid buffer
- Column: Shimadzu® shim-pack Velox SP-C18, 1.8 µm (100 x 2.1 mm).
- LC settings are as follows:

Mobile Phase gradient A:B flowing rate 0.4 mL/min from 0.00 to 2.59 minutes; 95% Mobile Phase B from 3.00 to 5.01 minutes; 25% Mobile Phase B from 5.01 to 5.30 minutes the oven temperature was 50°C for balancing columns.
- MS settings are as follows:

positive electrospray ionization mode by setting these followings:
Nebulizing Gas Flow at 3 L/min,
Heating Gas Flow at 10 L/min,
Interface Temperature at 300 °C,
Desolvation Line (DL) at 526°C,
Heat Block at 400°C, Drying Gas Flow at 10 L/min on a Multiple Reaction Monitoring (MRM) mode. (Table 1)
- Labsolution Software® was employed for a chromatogram analysis.

Mass spectrum

The mass spectra in MRM for identifying mitragynine and mitragynine-D3 are as follows: Mitragynine precursor ion (399.05), product ion 1 (173.95) and product ion 2 (159.0). Mitragynine-D3

precursor ion (402.1), product ion 1 (177.0) and product ion 2 (238.1).

Sample preparation

Ninety µL of mitragynine standard solution into blood from the blood bank to get the final sample volume of 18 mL. The concentrations of mitragynine in the sample was 500 ng/mL.

Three types of common blood collection tubes in this study were sodium fluoride/potassium oxalate, ethylenediaminetetraacetic acid (EDTA), and non-anticoagulant tubes.

Put 1 mL of the prepared sample into each blood collection tube. The samples were stored in the refrigerator at 4°C.

Determination the concentrations of mitragynine in the sample in each blood collection tube after the storage at one day, three days, five days, seven days, fourteen days, and thirty days were performed. The measurement of mitragynine concentrations on each determination was repeated for six times. The averages of the concentrations were used for statistical calculation.

Sample extraction [13]

'Modified QuEChERS'[14] was used as follows:

One hundred µL of the sample from the blood collection tube and 20 µL of mitragynine-D3 were put into a 1 mL Eppendorf® tube. Aacetonitrile (-20°C) was added into the tube until the mixture was up to 320 µL. Then, it was mixed with a vortex and was frozen at - 20°C for 10 minutes. Then it was set aside at the room temperature for 10 minutes. Forty mg of QuEChERS (UCT®) powder was put into the tube, mix with a shaker machine at 5,000 rpm for 5 minutes, and centrifuge at 15,000 rpm speed for 10 minutes at 4°C. Fifty µL of the centrifuged supernatant fluid was put into a 1 mL vial tube. One hyndred and fifty µL of mixture of mobile phase was added (A:B, 1:3) for the LC-MS/MS analysis.

Statistical analysis

Mean, standard deviation (SD) and repeated ANOVA were analyzed by SPSS version 22.0. A P - value < 0.05 was considered as statistically significant.

Results

Mitragynine and mitragynine-D3 could be identified by LC-MS/MS at about 3.15 minutes after injection as shown in the chromatogram in Figure 1.

The study showed that the differences in mitragynine concentrations in sodium fluoride/potassium oxalate tube on day 1 and day 3 compared with day 14 were statistically significant ($P < 0.05$). The difference of mitragynine levels on day 7 compared with day 14 was also statistically significant ($P < 0.05$) (Figure 2 and Table 1).

The EDTA tubes revealed that the differences in mitragynine concentrations on day 1 compared with day 7 and differences on day 3 and day 14 were statistically significant ($P < 0.05$) (Figure 3 and Table 2).

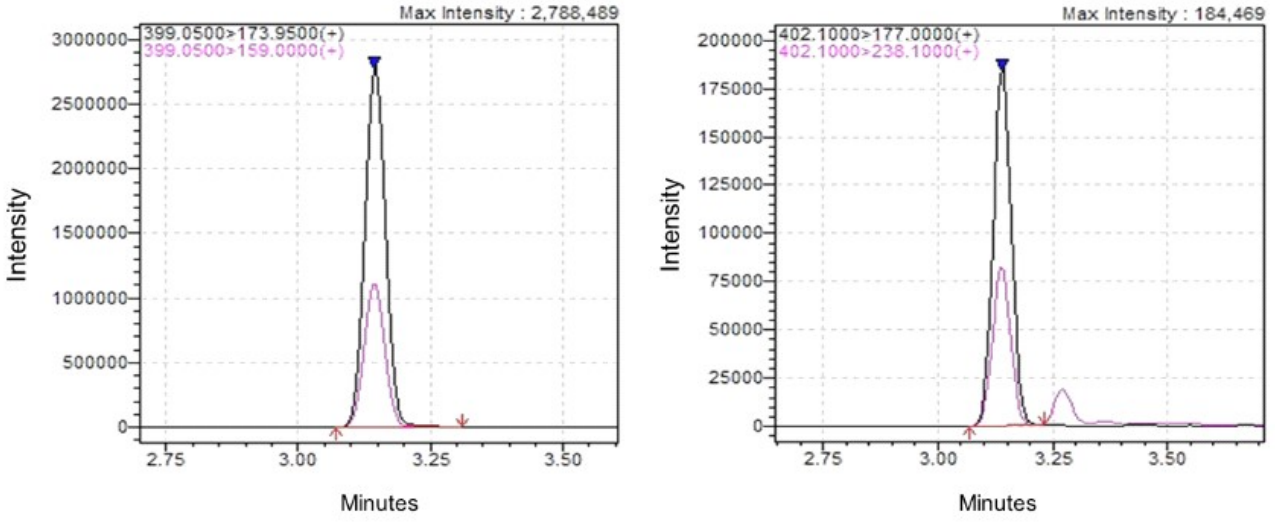

Figure 1. Chromatogram of mitragynine (left) and mitragynine-D3 (right).

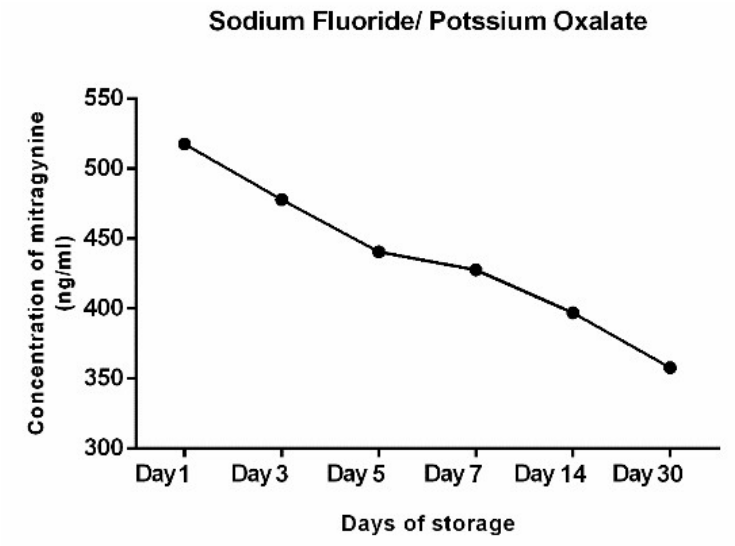

Figure 2. Average of mitragynine concentrations over 30 days in sodium fluoride/potassium oxalate tube.

Table 1. Statistical comparison of mitragynine concentrations in sodium fluoride/potassium oxalate tubes.

Anti-coagulant	Day		SD	*P*-value
NaF/Kox	Day 1	Day 3	15.075	0.941
		Day 5	17.036	1.0
		Day 7	12.767	0.192
		Day 14	12.874	<0.001
		Day 30	11.881	<0.001
	Day 3	Day 5	19.047	1.0
		Day 7	13.381	1.0
		Day 14	15.181	0.009
		Day 30	7.991	<0.001
	Day 5	Day 7	19.324	0.753
		Day 14	21.458	0.004
		Day 30	18.213	<0.001
	Day 7	Day 14	14.028	0.01
		Day 30	14.546	<0.001
	Day 14	Day 30	13.803	0.14

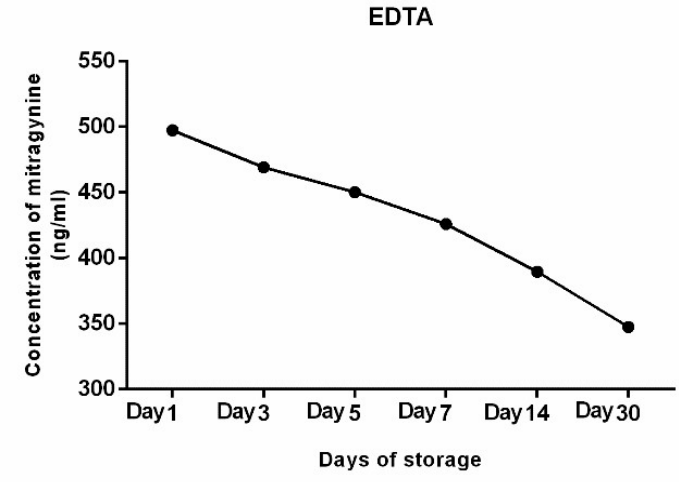

Figure 3. Average of mitragynine concentrations over 30 days in EDTA tube.

Table 2. Statistical comparison of mitragynine concentrations in EDTA tubes.

Anti-coagulant	Day		SD	*P* - value
EDTA	Day 1	Day 3	15.075	1.0
		Day 5	17.036	0.214
		Day 7	12.767	0.001
		Day 14	12.874	<0.001
		Day 30	11.881	<0.001
	Day 3	Day 5	19.047	1.0
		Day 7	13.381	0.085
		Day 14	15.181	0.001
		Day 30	7.991	<0.001
	Day 5	Day 7	19.324	1.0
		Day 14	21.458	0.194
		Day 30	18.213	0.001
	Day 7	Day 14	14.028	0.304
		Day 30	14.546	0.001
	Day 14	Day 30	13.803	0.122

No anticoagulant tubes demonstrated that the difference in mitragynine concentrations on day 1 and day 7 and differences on day 3 and day 7 compared with day 14 were statistical significance ($P < 0.05$) (Figure 4 and Table 3).

When comparing the mean concentrations of mitragynine in the three blood collection tubes in each storage period, there was no statistical significance ($P > 0.05$) (Figure 5 and Tables 4, 5).

The concentrations of mitragynine decreased with the time of storage in all types of blood collection tubes (Table 6). The differences of mean concentrations of all tubes were as follows: day 1 and day 7 49.3 ng/mL, day 1 and day 14 72.39 ng/mL, and day 1 and day 30 100.79 ng/mL.

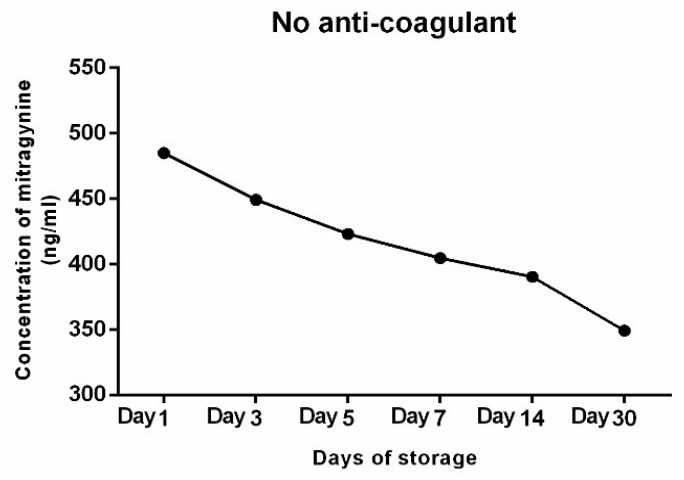

Figure 4. Average of mitragynine concentrations over 30 days in none anti-coagulant tube.

Table 3. Statistical comparison of mitragynine concentrations in none anti-coagulant tubes.

Anti-coagulant	Day		SD	*P*-value
None	Day 1	Day 3	15.075	0.153
		Day 5	17.036	0.068
		Day 7	12.767	0.016
		Day 14	12.874	<0.001
		Day 30	11.881	<0.001
	Day 3	Day 5	19.047	1.0
		Day 7	13.381	1.0
		Day 14	15.181	0.017
		Day 30	7.991	<0.001
	Day 5	Day 7	19.324	1.0
		Day 14	21.458	0.591
		Day 30	18.213	0.003
	Day 7	Day 14	14.028	0.025
		Day 30	14.546	<0.001
	Day 14	Day 30	13.803	0.185

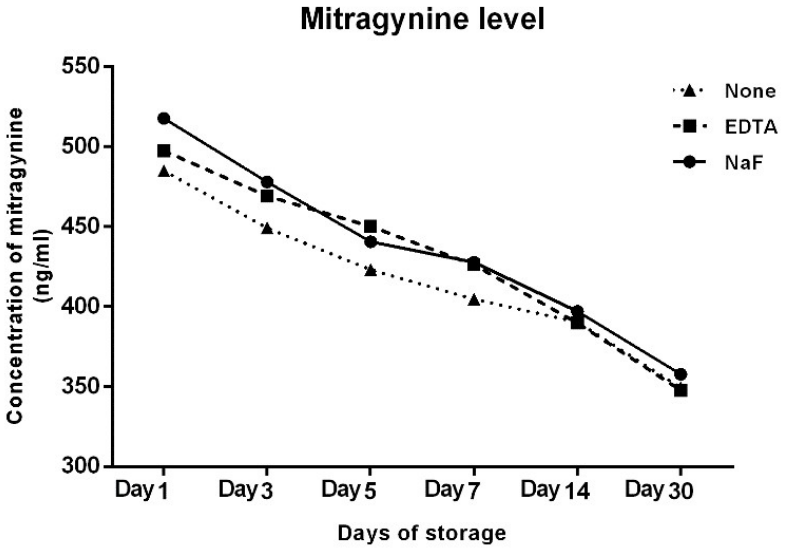

Figure 5. Average of mitragynine concentration across conditions over 30 days.

Table 4. Statistical comparison of the concentrations of mitragynine in each interval of all three tubes.

Day	Group		SD	*P*-value
	EDTA	NaF/Kox	13.251	0.717
Day 1		none	13.251	0.419
	NaF/Kox	none	13.251	0.249
	EDTA	NaF/Kox	14.231	0.442
Day 3		none	14.231	0.37
	NaF/Kox	none	14.231	0.894
	EDTA	NaF/Kox	26.000	0.858
Day 5		none	26.000	0.133
	NaF/Kox	none	26.000	0.97
	EDTA	NaF/Kox	16.208	0.152
Day 7		none	16.208	0.156
	NaF/Kox	none	16.208	0.988
	EDTA	NaF/Kox	11.793	0.543
Day 14		none	11.793	0.956
	NaF/Kox	none	11.793	0.58
	EDTA	NaF/Kox	11.468	0.39
Day 30		none	11.468	0.893
	NaF/Kox	none	11.468	0.466

Table 5. Statistical comparison of the concentrations of mitragynine in each successive period in all three tubes.

Anti-coagulant	Day		SD	*P* - value
EDTA	Day 1	Day 3	15.075	1.0
		Day 5	17.036	0.214
		Day 7	12.767	0.001
		Day 14	12.874	<0.001
		Day 30	11.881	<0.001
NaF	Day 1	Day 3	15.075	0.941
		Day 5	17.036	1.0
		Day 7	12.767	0.192
		Day 14	12.784	<0.001
		Day 30	11.881	<0.001
None	Day 1	Day 3	15.075	0.153
		Day 5	17.036	0.068
		Day 7	12.767	0.016
		Day 14	12.874	<0.001
		Day 30	11.881	<0.001

Table 6. Mean, standard variation and coefficient of variation of mitragynine concentrations in blood collection tubes at day 1 to day 30.

Tube		Day 1	Day 3	Day 5	Day 7	Day 14	Day 30
NaF/potassium oxalate	Mean	502.30	532.33	445.47	456.46	428.02	402.50
	SD	16.79	33.54	24.66	19.77	27.77	26.27
	CV	3.34	6.30	5.54	4.33	6.49	6.53
EDTA	Mean	497.41	545.44	450.21	426.14	420.10	392.17
	SD	11.49	34.15	24.13	21.25	22.50	9.95
	CV	2.31	6.26	5.36	4.99	5.36	2.54
No anticoagulant	Mean	486.40	530.08	485.98	455.60	420.81	389.07
	SD	34.15	13.83	62.40	36.61	13.54	16.82
	CV	7.02	2.61	12.84	8.04	3.22	4.32

Discussion

From this study, the concentrations of mitragynine in blood collection tubes stored at 4°C decreased up to 100 ng/mL (20.0% from the start concentration) in day 30 of the storage. And the statistical analysis found that the storage of blood samples in the blood collection tubes with or without anti-coagulant had no statistical significance when they were compared in each test period. However, comparing the stability of mitragynine in each tube, it was found that sodium fluoride/potassium oxalate tube was better than the others in which the mitragynine concentration was stable for at least 7 days while the others was stable for 5 days. The difference of mitragynine concentrations was statistical significance when the concentrations of mitragynine in sodium fluoride/ potassium oxalate tube on day 1 and day 14 were compared while the others had statistically significant on day 1 and day 5. This result is different from the research of Donna M, *et al*. [15] She reported that blood storage with spiked mitragynine 15 ng/ML in sodium fluoride/ potassium oxalate tube store at 4°C refrigerator and extracted by Liquid-Liquid extraction technique, had the stability of mitragynine until 30 days. The research by Busardo FP, *et al*. [16], demonstrated that the stability of the tube containing mephedrone in sodium fluoride/potassium oxalate tube could remain the best stability. However, different research results may arise from many factors involving in the pre-analysis phase (Pre-analytic variability); the initial concentration of mitragynine, sample preparation, sample extraction and sample preservation. In this research, the samples were preserved in the refrigerator that is routinely used in the laboratory.

This might cause the cooling efficiency of the refrigerator, but it could reflect the normal use in the laboratory. The concentration of mitragynine used in this study is also different from others which is another important factor that would lead to different results. Another factor of varying results is the difference in the instruments used in the determination of mitragynine concentration in the blood.

The limitations of this study were that there was no determination of mitragynine concentration at the start of the study (day 0) and no freeze-thaw study was done since the limitation of the standard and internal standard solution.

Conclusion

In this study, the stability of spiked mitragynine blood in blood collection tubes which were kept in a refrigerator at 4°C had no significant difference among the three types of blood collection tubes. However, we recommend to use sodium fluoride/ potassium oxalate tube for mitragynine determination since it showed mitragynine stability for at least 7 days. According to guidelines for collection of biological samples for clinical and forensic toxicological analysis,[17] if the blood sample could not be analyzed in 7 days, the sample should be stored at - 20°C or - 80°C. However, the study of the stability of mitragynine in blood kept at - 20°C or - 80°C should be further studied to confirm the appropriate storage of mitragynine in blood sample.

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Conflict of interest

The authors, hereby, declare no conflict of interest.

References

1. European Monitoring Centre for Drugs and Drug Addiction. EMCDDA-Europol 2012 Annual report on the implementation of council decision 2005/387/ JHA [Internet]. 2013 [cited 2019 Jul 14]; Available from: http://www.emcdda.europa.eu/publications/ implementation-reports/2012_en
2. United Nations Office on Drug and Crime. UNODC annual appeal 2017 [Internet]. [cited 2019 Jul 14]; Available from: https://www.unodc.org/unodc/en/ about-unodc/annual-report.html
3. Thanyarak Hospital statistics of drug abusers at Thanyarak Therapy Center for 2017 and 2018 fiscal years [Internet]. [cited 2019 Jul 14];Available from: http://www.pmnidat.go.th/thai/index.php?option= com_content&task=view&id=2738&Itemid=53
4. Feng LY, Battulga A, Han E, Chung H, Li JH. New psychoactive substances of natural origin: A brief review. J Food Drug Anal 2017;25:461-71.
5. Warner ML, Kaufman NC, Grundmann O. The pharmacology and toxicology of kratom: from traditional herb to drug of abuse. Int J Legal Med 2016;130:127-38.
6. Lydecker AG, Sharma A, McCurdy CR, Avery BA, Babu KM, Boyer EW. Suspected adulteration of commercial kratom products with 7-hydroxy-mitragynine. J Med Toxicol 2016;12:341-9.
7. Holler JM, Vorce SP, McDonough-Bender PC, Magluilo J Jr, Solomon CJ, Levine B. A drug toxicity death involving propylhexedrine and mitragynine. J Anal Toxicol 2011;35:54-9.
8. Neerman MF, Frost RE, Deking J. A drug fatality involving Kratom. J Forensic Sci 2013;58:278-9.
9. McIntyre IM, Trochta A, Stolberg S, Campman SC. Mitragynine 'Kratom' related fatality: a case report with postmortem concentrations. J Anal Toxicol 2015; 39:152-5.
10. Parthasarathy S, Ramanathan S, Ismail S, Adenan MI, Mansor SM, Murugaiyah V. Determination of mitragynine in plasma with solid-phase extraction and rapid HPLC-UV analysis, and its application to a pharmacokinetic study in rat. Anal Bioanal Chem 2010; 397:2023-30.
11. Kowalczuk AP, Lozak A, Zjawiony JK. Comprehensive methodology for identification of Kratom in police laboratories. Forensic Sci Int 2013; 233:238-43.
12. Fu H, Cid F, Dworkin N, Cocores J, Shore G. Screening and Identification of Mitragynine and 7-Hydroxymitragynine in Human Urine by LC-MS/MS. Chromatography 2015; 2:253-64.
13. Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. J Anal Toxicol 2013;37:452–74.
14. Major RE. QuEChERS - A new technique for multiresidue analysis of pesticides in foods and agricultural samples. LCGC Asia Pacific 2008;11:1-7.
15. Papsum DM, Chan-Hosokawa A, Friederich L, Brower J, Graf K, Logan B. The trouble with kratom:

Analytical and interpretative issues involving mitragynine. J Anal Toxicol 2019;43:615-29.

16. Busardo FP, Kyriakou C, Tittarelli R, Mannocchi G, Pantano F, Santurro A, et al. Assessment of the stability of mephedrone in ante-mortem and post-mortem blood specimens. Forensic Sci Int 2015;256: 28-37.
17. Dinis-Oliveira RJ, Viera DN, Magalhaes T. Guidelines for collection of biological samples for clinical and forensic toxicological analysis. Forensic Sci Res 2016; 1:42-51.