APPLICATION NOTE 22062

Simple quantitation of kratom components from urine by LC-MS/MS for forensic toxicology

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Goals

To achieve accurate and precise liquid chromatography with tandem mass spectrometry (LC-MS/MS) quantitation of two opioid-receptor agonists and major alkaloid constituents of the *Mitragyna speciosa* (kratom) plant in human urine. To develop an extraction using polymeric SPE coupled to a simple and reliable chromatographic separation.

Introduction

Mitragyna speciosa, also known as kratom or ketum, is a tropical evergreen tree in the coffee family and is indigenous to Southeast Asia.[1] Historically, the leaves of the plant are chewed, or dried and brewed as tea, to release a plethora of indole and oxindole alkaloids that exhibit sedative, analgesic, and stimulant-like effects.[2] In traditional communities, it has also been used as an aid for withdrawal from opioid dependence and also fatigue in farm workers for many years.[3] While the cultivation and selling of the plant has been outlawed in countries, such as Thailand, the powdered material is now available to purchase online from across the world, including Europe and North America.[2,4] The influx of this recreational drug into new territories means that *Mitragyna* alkaloids are not usually within scope of traditional forensic toxicology assays.

The US Poison Control Center reports a greater than 50-fold increase in calls regarding Kratom from 2011 to 2017, and more recently, a six-fold increase in just two years (from 2016 to 2018).[5,6]

Kratom is an unscheduled opioid, which acts on the opioid receptors, leading to abuse and dependence. It has recently been associated with multiple deaths and

is suspected to be adulterated with other drugs. Due to its potential for abuse and addiction, both the Drug Enforcement Agency (DEA) and the FDA are striving together to get a Schedule I designation for Kratom. It is currently listed by the DEA as a "drug of concern."

Mitragynine (MG) and 7-hydroxymitragynine (OH-MG) are major psychoactive compounds found in the plant leaves, and there are currently some existing methods of analysis for the former. OH-MG, however, is notably unstable unless stored in a basic environment, preferably at -20 °C.[7]

In this application note, a complete workflow is described to quantify MG and OH-MG from human urine. It comprises of a sample extraction procedure using ammonia with a Thermo Scientific™ HyperSep™ Retain PEP Solid Phase Extraction (SPE) plate that lends itself well to the stability requirements of OH-MG, making the process seamless and straightforward. Furthermore, the simple reverse-phased chromatography afforded by the Thermo Scientific™ Hypersil GOLD™ VANQUISH™ UHPLC 1.9 µm column allows for adequate retention and isolation of each compound prior to tandem mass spectrometry (MS/MS) analysis with the Thermo Scientific™ TSQ Quantiva™ triple-stage quadrupole mass spectrometer.

The method was assessed intra-day to determine linearity, accuracy and precision, carryover, specificity, recovery, and matrix effects, as presented in Table 1.

Table 1. Summary of assay

Parameter	Value	
Analytes	Mitragynine (MG)	7-hydroxymitragynine (OH-MG)
Analytical matrix	Human urine	
Calibration range	15.0–1000 ng/mL	
Lower limit of quantification (LLOQ)	15.0 ng/mL	
Calibration model	Linear regression, R^2>0.996	
Weighting factor	$1/x^2$	
Carryover	None observed	
Specificity	Acceptable	
Accuracy (bias) and precision	-0.2–6.6% (CV% 1.6–5.1)	-2.3–9.2% (CV% 3.0–9.3%)
Recovery	87.4%	90.3%
Matrix factor (IStd normalized)	0.969 (CV% 11.3)	1.01 (CV% 0.9)

Experimental

Instrumentation

- Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of the following:
  - System base Vanquish Horizon (P/N VH-S01-A)
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Active pre-heater (P/N 6732.0110)
- TSQ Quantiva triple-stage quadrupole mass spectrometer
- Thermo Scientific™ HyperSep™ Universal Vacuum Manifold (P/N 60104-230)
- Thermo Scientific™ HyperSep™ Glass Block Vacuum Manifold Pump (P/N 60104-241)

Consumables

- Thermo Scientific™ Hypersil GOLD™ VANQUISH™ C18 UHPLC Column, 1.9 µm, 2.1 x 100 mm (P/N 25002-102130-V)
- Thermo Scientific™ HyperSep™ Retain PEP 30 mg/2 mL 96 fixed well plate, 1 pk (P/N 60306-207)
- Thermo Scientific™ WebSeal™ 96-well non-coated certified polypropylene plates (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ nonsterile silicone/PTFE mats, blue, 5 pack (P/N 60180-M122)

Reagents

- Thermo Scientific™ UHPLC-MS grade water (P/N W8-1)
- Thermo Scientific™ UHPLC-MS grade methanol (P/N A456-1)
- Fisher Chemical™ Optima™ UHPLC-MS grade formic acid (P/N A117-50)
- Fisher Chemical™ analytical grade ammonia solution (35%, v/v) (P/N A/3280/PB15)

Sample preparation protocol

Pure methanol was used to prepare individual stock solutions of MG, OH-MG, MG-D_3, and OH-MG-D_3 (100 µg/mL). Working standards containing both analytes of interest, 7-OH-MG and MG, were prepared in ammoniated methanol (2%) in the range of 0.150 to 10.0 µg/mL. Blank human urine from a single source was fortified one-in-ten with these working solutions to produce eight calibration standards in the range of 15.0 to 1000 ng/mL. Quality control samples were prepared from separate working solutions and spiked into human urine to produce final concentrations of 15 ng/mL (LLOQ), 40 ng/mL (LQC), 300 ng/mL (MQC), and 800 ng/mL (HQC). The internal standard solution consisted of 7-OH-MG-D_3 and MG-D_3 in MeOH/NH_3 (98/2, v/v). Samples were fortified to produce a matrix concentration of 50 ng/mL. All working solutions were stored at -20 °C.

Solid phase extraction procedure

1. Add 100 µL of urine to a 96-well plate.
2. Add 10 µL of internal standard solution.
3. Add 500 µL of water.
4. Condition HyperSep Retain PEP 30 mg plate with 1 mL of methanol.
5. Equilibrate HyperSep Retain PEP 30 mg plate with 1 mL of water.
6. Transfer samples to HyperSep Retain PEP 30 mg plate.
7. Wash HyperSep Retain PEP plate with 500 µL of 2% ammonia in methanol/water (5/95, v/v). Discard to waste.
8. Wash HyperSep Retain PEP plate with 500 µL of 2% ammonia in methanol/water (50/50, v/v). Discard to waste.
9. Elute HyperSep Retain PEP plate with 2 x 250 µL of methanol. Keep the sample eluate.
10. Evaporate sample eluate to dryness under nitrogen at 50 ˚C.
11. Reconstitute with 100 µL of methanol/water (25/75, v/v).
12. Cap, vortex, and centrifuge sample prior to analysis.

Chromatography and separation conditions

Table 2. UHPLC conditions

Parameter	Value
Mobile phase A	Water/formic acid (100/0.1, v/v)
Mobile phase B	Methanol
Flow rate	0.5 mL/min
Run time	6.5 min
Column temperature	60 °C, active preheating and still air mode
Injection volume	10 µL

Table 3. LC gradient

Time (min)	B % Methanol
0.0	25
0.5	25
3.0	85
3.0	100
4.0	100
4.0	25
6.5	25

Mass spectrometry conditions

Table 4. MS/MS source parameters

Parameter	Value
Source	Thermo Scientific™ Ion Max source with HESI-II probe
Polarity	Positive ionization
Spray voltage	3500 V
Vaporizer temperature	400 °C
Sheath gas pressure	50 Arb
Aux gas pressure	15 Arb
Ion transfer tube temperature	350 °C
CID gas pressure	1.5 mTorr

Table 5. Compound transition details

Compound	Abbreviation	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	Retention time (min)
Mitragynine	MG	Positive	399.2	174.1	31.7	2.31
Mitragynine-D_3	MG-D_3	Positive	402.3	177.2	30.7	2.30
7-Hydroxymitragynine	OH-MG	Positive	415.2	190.1	28.8	1.81
7-Hydroxymitragynine-D_3	OH-MG-D_3	Positive	418.3	193.2	30.2	1.81

Table 6. Diverter valve settings. Position 1-2 directs to waste. Position 1-6 directs to mass spectrometer for analysis.

Time (min)	Position
0.00	1-2
1.65	1-6
1.95	1-2
2.15	1-6
2.45	1-2

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.10, was used for liquid chromatography mass spectrometry (LC-MS) system control, data acquisition, and data analysis.

Results and discussion

Calibration model, linearity, and range

The calibration model was established using eight non-zero calibrators (15, 40, 70, 80, 90, 100, 800, and 1000 ng/mL) analyzed in duplicate intra-batch. Calibration standards were freshly prepared from working solutions in MeOH/NH_3 (98/2, v/v) and subjected to solid phase extraction. The calibration model for both MG and OH-MG was produced using a least squares linear regression with $1/x^2$ weighting. The coefficients of determination were above 0.996 for both analytes.

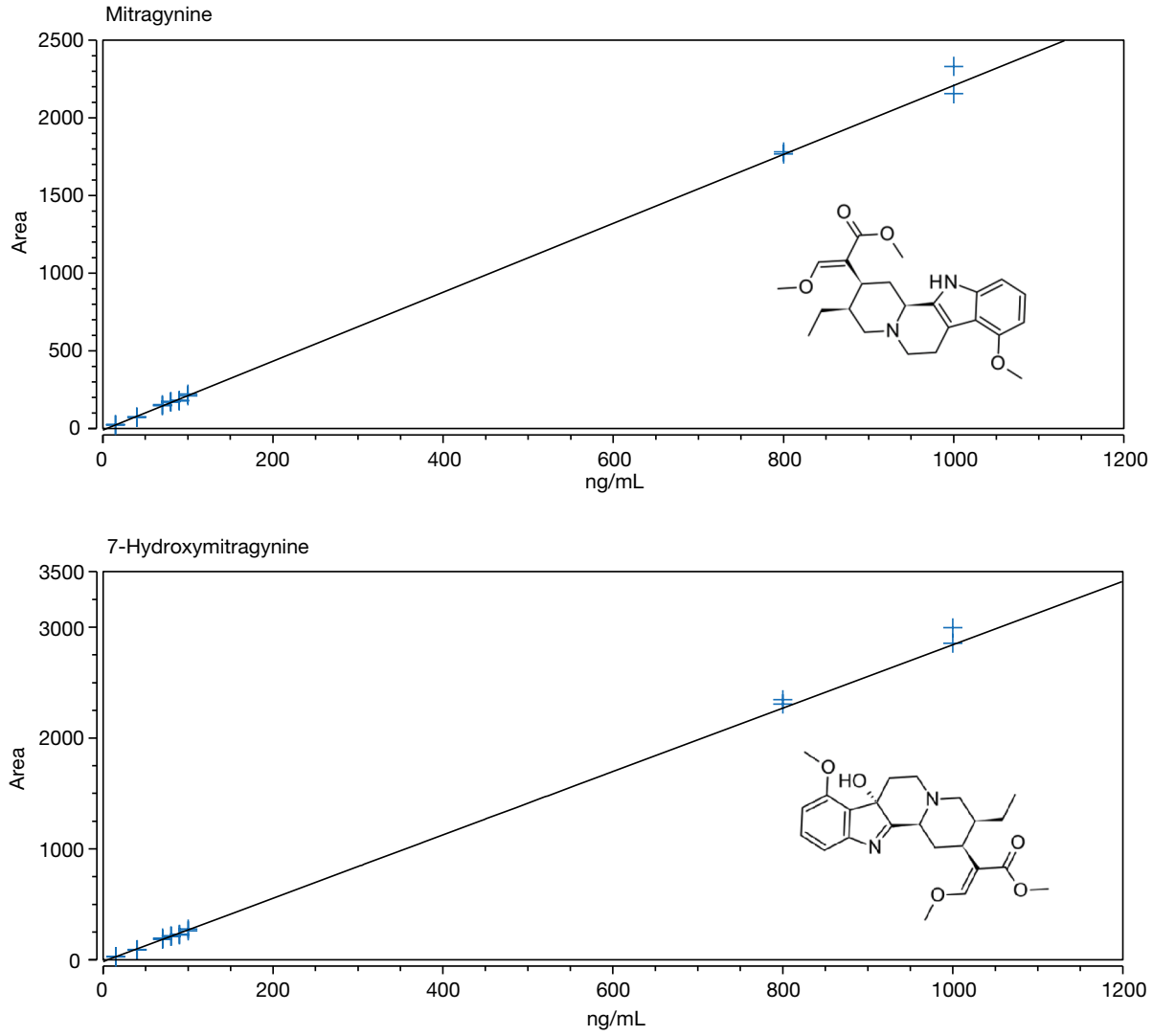

Figure 1. Calibration model for mitragynine and 7-hydroxymitragynine

Accuracy and precision

Accuracy and precision (A&P) were evaluated via analysis of quality control (QC) samples in replicate (n=6) at four concentration levels over the calibration range. These tested concentrations were 15 ng/mL (LLOQ), 40 ng/mL (LQC), 300 ng/mL (MQC), and 800 ng/mL (HQC) for both analytes. Accuracy was reported as bias (%) according to criteria of ±15% for low, mid, and high QC levels and ±20% for LLOQ level, while precision was assessed under the same criteria as the coefficient of variation (CV %). Intra-batch A&P was acceptable for both analytes with bias <9.2% and CV <9.3% at each of the four QC levels, as shown in Table 7.

Table 7a. Accuracy and precision for mitragynine

	Mitragynine (ng/mL)			
	LLOQ 15.0	Low 40.0	Medium 300	High 800
Batch 01	16.5	40.4	305	854
	16.5	37.5	310	867
	15.5	41.2	303	814
	15.8	42.3	302	809
	16.0	37.4	297	770
	15.7	40.7	309	809
Mean	16.0	39.9	304	820
Bias %	6.6	-0.2	1.4	2.5
CV %	2.7	5.1	1.6	4.3

Table 7b. Accuracy and precision for 7-hydroxymitragynine

	7-hydroxymitragynine (ng/mL)			
	LLOQ 15.0	Low 40.0	Medium 300	High 800
Batch 01	16.0	39.2	315	890
	15.0	36.5	309	1024
	15.1	40.4	304	812
	14.6	41.1	310	811
	14.7	38.7	293	878
	15.0	38.8	319	826
Mean	15.1	39.1	308	874
Bias %	0.4	-2.3	2.8	9.2
CV %	3.4	4.1	3.0	9.3

Specificity

Blank matrix from six individual sources was extracted as double blank samples (no analyte nor internal standard added) along with single blank samples of one source (internal standard added only) and an upper limit of quantification (ULOQ) sample (no internal standard). All were investigated for specific matrix-related interferences of the same mass transition and retention window as the analytes. Any interference was far below the 5% (IS concentration) and 20% (analyte LLOQ concentration) peak area threshold set by regulatory criteria. As such, specificity was acceptable. Figure 2 shows a representative chromatogram of mass components.

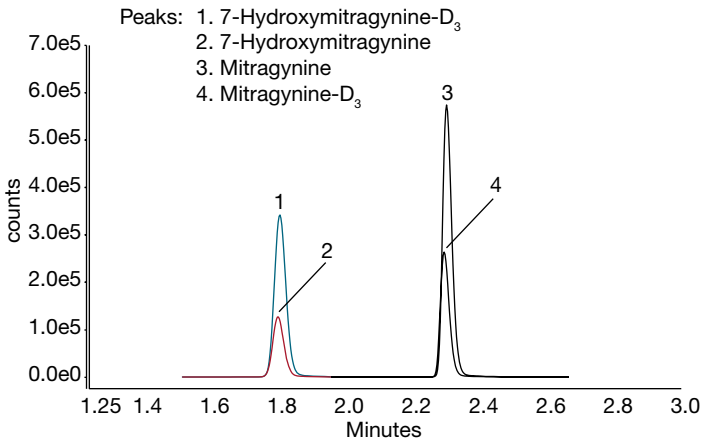

Figure 2. Representative chromatogram at 100 ng/mL

Carryover

The carryover was assessed with the injection of two double blank samples following an injection of the highest calibration sample (ULOQ). The area response of the analyte and internal standard in the blank samples was compared to the area responses of the LLOQ sample after them. No detectable carryover was present using the Vanquish Horizon UHPLC system.

Matrix effects

Extended exposure of OH-MG to acidic environments renders the analyte unstable and thus a load/wash procedure in base was employed. At pH 10, the logD values of OH-MG and MG are 2.7 and 3.3, respectively, while both remain uncharged at such a high pH. The HyperSep Retain PEP sorbent comprises a polystyrene divinylbenzene (DVB) material modified with urea functional groups. This allows for versatile, balanced retention of polar and non-polar analytes. Here, the expected retention mechanism for the alkaloids is a non-polar interaction with

the PEP sorbent that is orthogonal to the reversed-phase chromatography used afterward. An extraction procedure with this chemistry in base is particularly selective when cleaning up from a mildly acidic matrix such as urine, where many matrix components will be conjugated, polar, or ionized at the native pH. Additionally, the polymeric-DVB backbone of the solid phase also demonstrates greater resistance to high pH than with silica-based sorbents. This makes the HyperSep Retain PEP plate an excellent choice for this application cleanup.

Matrix effects were evaluated from a single source of human urine. Six extracted double blank samples were fortified post-extraction with a standard solution of analyte and internal standard at the fourth calibrator level (80 ng/mL). The mean analyte peak area from these samples was compared against the mean analyte peak area of non-extracted samples of the same concentration in standard solution. The resulting analyte matrix factor was normalized with the equivalent IS response to deliver an internal standard normalized matrix factor of 0.969 (CV% 11.3) and 1.01 (CV% 0.9) for MG and OH-MG, respectively. Representative data is shown in Table 8.

Recovery

The assay recovery was assessed at the fourth calibrator level by separately preparing six extracts at 80 ng/mL. Recovery was calculated by comparing the mean analyte peak area of each analyte in the extracts to the mean analyte peak area of the same blank urine source fortified post-extraction with compound and internal standard. Figure 3 shows recovery data for MG and OH-MG.

While no specific acceptance criteria were applied, recovery using the HyperSep Retain 30 mg plate was high: 87.4% and 90.3% for MG and OH-MG, respectively.

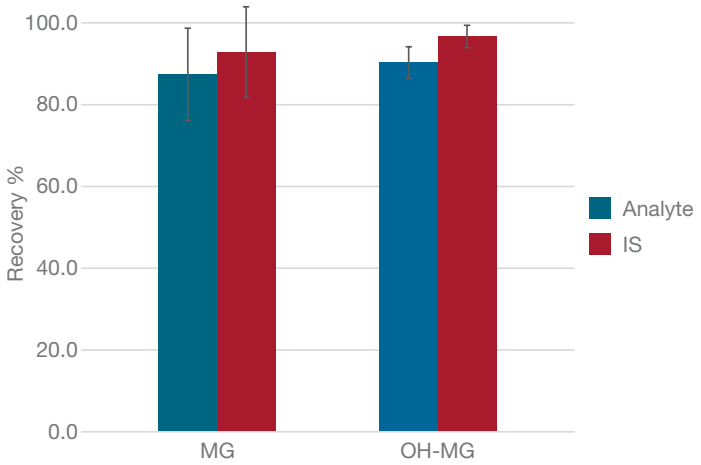

Figure 3. Extraction recovery of MG and OH-MG

Conclusion

- Negligible interference from matrix effects thanks to thorough sample cleanup with the HyperSep Retain PEP well plates and selective chromatography on the Hypersil GOLD VANQUISH column
- Fast, easy and robust separation of two major psychoactive alkaloids from the kratom plant in human urine for toxicology and research applications
- High recovery of both compounds thanks to the simple and robust sample extraction procedure using HyperSep Retain PEP well plates from only 100 µL urine
- Accurate and precise quantitation combining the separation speed and robustness of the Hypersil GOLD VANQUISH column and the Hypersil Retain PEP 96-well plate with the power of the Vanquish Horizon UHPLC system, TSQ Quantiva triple-stage quadrupole MS/MS system, and Chromeleon CDS with advanced MS data processing

Table 8. Matrix effect data for mitragynine and 7-hydromitragynine. Overspike samples, n=6. Non-extracted samples, n=3.

	Mean analyte response	Analyte matrix factor	Mean IS response	IS matrix factor	IS normalized matrix factor
MG (overspike)	1268412	0.985	821589	1.02	0.969
MG (non-extracted)	1287674		808479		
OH-MG (overspike)	892777	1.01	470094	1.00	1.01
OH-MG (non-extracted)	887150		470268		

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