The Optimized Separation and identification of Kratom Alkaloids using High-Res Mass Spectrometry

Stephanie Basiliere [1]***, BS*; Kelsie Bryand*** [1]***, MS; Sarah Kerrigan***[1]***, PhD***

[1] *Department of Forensic Science, Sam Houston State University, Huntsville, TX , 77340*

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ABSTRACT

Mitragynine (9-methoxycorynantheidine, Kratom) and 7-hydroxymitragynine are naturally occurring corynanthe-type indole alkaloids present in the leaves of *Mitragyna speciosa*. This flowering plant of the *Rubiaceae* genus contains more than twenty alkaloids, of which mitragynine (MG) is the principal pharmacologically active component with 7-hydroxymitragynine (MG-OH) being a minor psychoactive constituent. Mitragynine and 7-hydroxymitragynine are μ-opioid agonists. Kratom also contains two diastereoisomers of mitragynine (speciociliatine, SC and speciogynine, SG) and another alkaloid paynantheine (PY). Although these three compounds are not known to be psychoactive, they are some of the most prevalent compounds found in kratom. During the development of an analytical method for MG, MG-OH, PY, SC, and SG in urine, a total of three different mobile phase additives were evaluated along with different ionization parameters and mobile phase gradients. These three additives were formic acid, ammonium formate, and ammonium acetate. Although kratom alkaloids were able to be identified in with all three additives present, there were differences in chromatography, solubility, and sensitivity that may impede identification with liquid chromatography time-of-flight mass spectrometry (LC-qTOF-MS). In addition, the fragmentation pathways for all analytes were investigated. The most abundant fragments for all compounds were associated with quinolizine ring (C-ring) cleavage and the loss of the substituted piperidine between C2 and C5. The abundance and specificity ultimately led to this being selected for quantitation for SC (399→174) , SG (399→174) and PY (397→174). Variations of quinolizine ring cleavage predominated for all other major product ions, as well as formation of intact substituted piperidine ions.

INTRODUCTION

Kratom is a psychoactive drug that comes from the leaves of *Mitragyna speciosa*, a tropical evergreen tree of the *Rubiaceae* family (1). Forty-four compounds have been isolated from the leaves of *M. speciosa* (2), many of which include both indole and oxindole alkaloids. Mitragynine and 7-hydroxymitragynine are the principal pharmacologically active compounds in kratom. While not psychoactive, speciociliatine, speciogynine, and paynantheine are also prevalent compounds found in kratom. Speciociliatine and speciogynine are both diastereoisomers of mitragynine while paynantheine is a dehydro analog of mitragynine (1).

Kratom elicits both stimulating and sedative effects in a dose-dependent manner (1). Low doses of kratom are reported to produce a stimulant effect similar to cocaine, and high doses are reported to produce opiate-like effects (2). In the western world, the drug is used recreationally for its euphoric effects. However, a growing number of individuals also use kratom as an opioid alternative for the treatment of chronic pain, or to treat opioid addiction. Both mitragynine and 7-hydroxymitragynine act as partial agonists at mu-opioid receptors and competitive antagonists at kappa and delta opioid receptors (2). Given the current epidemic of opioid abuse in the United States, non-medical use of kratom is of growing concern.

Identification of kratom alkaloids in biological samples presents a significant challenge in terms of analytical detection. LC-qTOF-MS is a high resolution MS technique that offers high sensitivity and significant benefits in terms of mass accuracy and structural identification. However, it is important to consider mobile phase additives during method development as it has a significant effect on separation and ionization. In addition, identifying fragmentation pathways and subsequent structural identification of ions is another crucial part of method development. During the development of an analytical method for these compounds in urine using LC-qTOF-MS, we identified the fragmentation pathways of the kratom alkaloids and explored the effects of common mobile phases compositions.

RESULTS

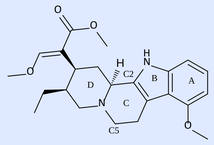
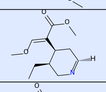
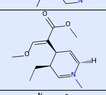
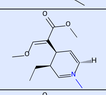
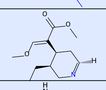
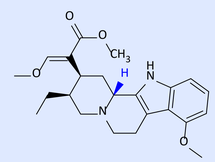
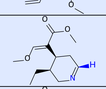
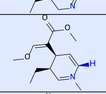
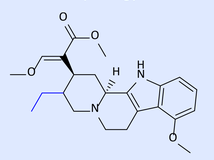
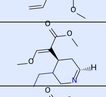
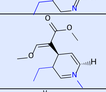
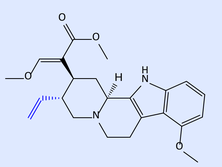
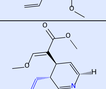
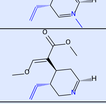
Compound	Structure	Molecular Formula	Accurate Mass	Exact Mass	PPM Shift
Mitragynine		$C_{11}NOH_{12}$	174.0911	174.0913	-0.1
		$C_{12}NO_3H_{20}$	226.1433	226.1436	-0.1
		$C_{13}NO_3H_{20}$	238.1431	238.1438	-0.3
7-Hydroxymitragynine		$C_{11}NO_2H_{12}$	190.0862	190.0863	-0.1
		$C_{13}NO_3H_{20}$	238.1434	238.1438	-0.2
		$C_{12}NO_3H_{20}$	226.1434	226.1436	-0.1
Speciociliatine		$C_{11}NOH_{12}$	174.0912	174.0913	-0.1
		$C_{12}NO_3H_{20}$	226.1434	226.1436	-0.1
		$C_{13}NO_3H_{20}$	238.1432	238.1438	-0.3
Speciogynine		$C_{11}NOH_{12}$	174.0909	174.0913	-0.2
		$C_{12}NO_3H_{20}$	226.1431	226.1436	-0.2
		$C_{13}NO_3H_{20}$	238.1430	238.1438	-0.3
Paynantheine		$C_{11}NOH_{12}$	174.0912	174.0913	-0.1
		$C_{13}NO_3H_{18}$	236.1275	236.1281	-0.3
		$C_{12}NO_3H_{18}$	224.1277	224.1281	-0.2

Table 1: Proposed fragmentation and mass accuracy of the Mitragyna alkaloids using positive electrospray ionization.

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MATERIALS AND METHODS

Chemicals

Reference standards for mitragynine, 7-hydroxymitragynine, mitragynine-D3, and 7-hydroxymitragynine-D3 were purchased at a concentration of 100 μg/mL from Cerilliant (Round Rock, TX). Reference standards for paynantheine and speciociliatine were purchased from Chromadex (Irvine, CA) and speciogynine was provided by the National Center for Natural Products Research (NCNPR) at the University of Mississippi, (Oxford, MS). Reference standards were purchased as methanolic standards with the exception of paynantheine, speciociliatine and speciogynine (solids). 7-Hydroxymitragynine, which is known to be unstable, was purchased in ammoniated methanol (1% concentrated ammonium hydroxide in methanol, v/v) and stored at -80ºC. All working standards or mixtures containing MG-OH (or its deuterated analog) were also prepared and stored accordingly, prior to use. Unless otherwise stated, solvents and inorganic reagents were LC or ACS grade, respectively. Acetonitrile and formic acid (LCMS grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate and ammonium formate (LCMS grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purified using a Millipore Direct-Q®UV Water Purification system (Billerica, MA, USA).

Instrumentation

Analysis was achieved using an Agilent 6530 Accurate Mass LC-Q/TOF-MS with a heated electrospray ionization (ESI) source. Chromatography was performed with an Agilent Poroshell EC-C18 Column (2.1 x 100 mm, 2.7 μm particle size) and an Agilent Poroshell 120 EC-C18 Guard Column (2.1 x 5 mm, 2.7 μm particle size). The method used multiple reaction monitoring mode (MRM) using a minimum of 3 transitions (2 qualifier ions and a quantitation ion for each compound). All data analysis was performed using Agilent MassHunter software.

DISCUSSION & CONCLUSIONS

Method development for the compounds in *M. speciosa* is a challenging due to the large number of structurally similar alkaloids and diastereoisomers found in the plant. LC-qTOF-MS and other high resolution MS techniques are particularly useful for complex analytes such as these. In addition, methods utilized during the analysis of forensic toxicological samples must be highly sensitive and selective.

During method development for LC-MS, mobile phase composition must be considered. 7-Hydroxymitragynine (m/z 415) has a tendency to form a very prominent water adduct (m/z 433), particularly when formic acid (0.1%) is used as a mobile phase additive. Adducts are relatively common in LC-MS analysis, but are known to reduce both sensitivity and reproducibility. Adduct formation was previously reported for Mitragyna alkaloids by Kikura-Hanajiri, who integrated the adduct into their method, rather than removing it (3). In our study, traditional means to eliminate the adduct by modifying source conditions were unsuccessful. In addition, solubility problems persisted with other additives. Mobile phase additives (formic acid, ammonium acetate, and ammonium formate) at different concentrations (5, 10, 50, 100 mM ammonium formate/acetate and 0.1% formic acid) and in combination were also evaluated. Using the fully optimized method, it was possible to completely eliminate the water adduct and solubility problems using 5mM ammonium acetate solution in water and acetonitrile.

In addition to mobile phase selection, identifying the fragmentation pathways can improve the selectivity of a method because it allows for highly specific precursor ion transitions to be used for identification and quantitation. A total of three transitions for each compound were selected from the MS spectra (**Table 1**) for this analytical method. These compounds fragment in an identical fashion, producing identical MS-MS spectra. The corynantheidine-type compounds undergo characteristic cleavage of the quinolizine ring (C-ring) to yield stable indole (m/z 174 and 190) and piperidine derivative fragments (m/z 238, 236, 226, 224). The 238 and 236 ions result from cleavage at C5, while 226 and 224 are formed via neutral loss of the methoxy indole group between C5 and nitrogen. High-resolution mass spectrometry allowed product ions to be structurally identified with mass accuracies within 0.5 ppm.