A Validated Method for the Determination of Salvinorin A and Salvinorin B in Forensic Toxicology Samples

Stephanie Basiliere, BS; Tracy Gastineau, MS; Sarah Kerrigan, PhD*
Department of Forensic Science, Sam Houston State University,
Huntsville, TX 77340

ABSTRACT

Salvia divinorum is a perennial plant from the Lamiaceae (mint) family found in the Sierra Mazateca region of Oaxaca, Mexico and has been used in religious and medicinal rituals for centuries. *Salvia divinorum* is also used as a recreational drug due to its profound hallucinogenic properties. As many as 35 countries have enacted legislation to control *S. divinorum* and/or Salvinorin A, its principal psychoactive component. Street names include Diviner's Sage, Maria Pastora, Sally-D, and Magic Mint. Salvinorin A and its major metabolite (Salvinorin B) are of forensic interest, but are rarely reported during routine toxicological testing. Salvinorin A is the only known naturally occurring, non-nitrogenous hallucinogen with a high affinity for the kappa-opioid receptor (KOR). Although it is not federally controlled, its rapid onset of action and powerful hallucinogenic effect contribute to its abuse potential.

We report an optimized and scientifically validated method for the determination of Salvinorin A and Salvinorin B in biological matrices. Solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC/MS) using selected ion monitoring (SIM) were used throughout. Both the extraction method and GC/MS parameters were optimized to achieve optimal chromatographic separation and detection. In the absence of a commercially available deuterated salvinorin at the time of the study, testosterone-D_3 was used as the internal standard. A GC inlet temperature of 250ºC and an initial oven temperature of 260ºC produced optimal results. The temperature program involved a 0.5 min hold at 260ºC with a ramp up to 290ºC with a 30ºC/min rate and a final hold for 17 minutes. The retention times were 6.9 min for Salvinorin B, and 7.9 min for Salvinorin A.

The method was evaluated using recommendations of the Scientific Working Group for Toxicology (Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology, 2013). Analytical recovery, limit of linearity, limit of detection, quantitation, precision, bias, carryover, interference, and dilution integrity were evaluated. Both Salvinorin A and Salvinorin B were linear from 0-1,000 ng/mL. The limits of detection and quantitation for Salvinorin A were 5 ng/mL and 10 ng/mL, respectively. The limit of detection for Salvinorin B was 20 ng/mL. Precision and bias were evaluated and quantitated at three concentrations and produced %CVs and bias of <20%. Carryover was not present at 1,000 ng/mL and no interferences were observed from common drugs of abuse. Interferences from other salvinorins and divinatorins were also evaluated. Dilution integrity was evaluated using biological matrices that were diluted 1:10 prior to solid phase extraction. These results also demonstrated acceptable precision and bias. This validated method provides an efficient and reliable method to quantitatively identify salvinorins of forensic interest in biological matrices using GC/MS.

INTRODUCTION

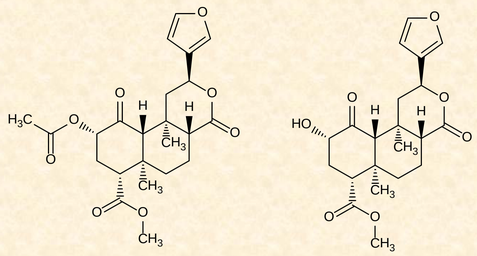
According to the National Survey on Drug Use and Health Report published by SAMHSA, it is estimated that 1.8 million persons aged 12 or older used *Salvia divinorum* in their lifetime and approximately 750,000 did so during the past year (SAMHSA, 2008). Drug use is reported to be most common among young adults (18-25 years olds), although as many as 2.5% of 10th graders and 4.4% of 12th graders report using "Salvia" during the past year.

The drug is domestically grown and imported from Mexico and South America. The Internet is used for promotion and distribution purposes. Although *Salvia divinorum* and Salvinorin A are not currently scheduled under the Federal Controlled Substances Act (CSA), several states including California, Delaware, Florida, Hawaii, Illinois, Kansas, Kentucky, Louisianna, Maine, Maryland, Minnesota, Missouri, Nebraska, North Carolina, North Dakota, Ohio, Oklahoma, Tennessee, Virginia and Wisconsin have enacted legislation to curb its use (DEA, 2013).

INTRODUCTION, Cont'd

Salvinorin A can evoke both hallucinogenic and depressant-like effects. These include vivid imagery, body and object distortions, dysphoria, uncontrolled laughter, depersonalization and overlapping realities. Adverse physical effects can include slurred speech, poor coordination and dizziness. Although relatively little is known of its metabolism, Salvinorin A can undergo hydrolysis (of the ester) for form Salvinorin B (Figure 1). Subjects who smoked 580 µg of the drug in 75 mg of dried salvia leaves over three minutes produced urinary concentrations of 2 to 11 ng/mL Salvinorin A. No parent drug was detect after 1.5 hours (Pichini, 2005). This suggests that both Salvinorin A and B should be targeted, and that analytical methods must have detection limits that are sufficiently low to be effective in forensic toxicology investigations. We describe a simple and effective procedure to detect the drug and metabolite in urine using solid phase extraction (SPE) and GC/MS.

Figure 1. Structures of Salvinorin A and B.



Salvinorin A Salvinorin B

MATERIALS AND METHODS

Reference materials and standards: Salvinorin A, Salvinorin B and testosterone-D_3 (internal standard) were purchased from Cerriliant (Round Rock, TX). Salvinorins A-G and Divinatorin A-C were also provided by Dr. Thomas Munro (McLean Hospital in Belmont, MA). Controls and calibrators were prepared from pooled human urine from drug-free volunteers.

Extraction: Cerex® Polychrom™ CLINII solid phase extraction columns were used to isolate Salvinorin A/B from urine. To 2 mL urine, 100 µL (50 ng/mL) of testosterone-D_3 was added and vortex mixed. Phosphate buffer (4 mL, pH 6.0) was added and samples were poured onto unconditioned SPE columns. Successive washes were performed using 1 mL aliquots of deionized water and 1 M acetic acid under gravity flow. Columns were dried for 5 min at full vacuum and again washed using 1 mL hexane. Salvinorin A and B were eluted in 1 mL of ethyl acetate and extracts were evaporated to dryness under nitrogen. Samples were reconstituted in 20 µL of ethyl acetate and transferred to autosampler vials for analysis. A total of 2 µL was injected on the GC/MS for analysis.

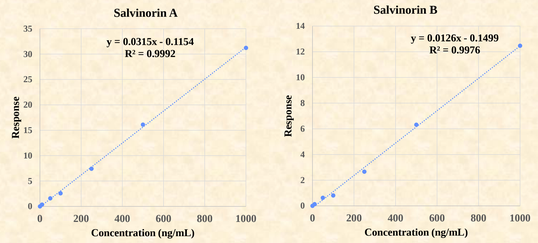
Instrumentation: GC-MS analysis was performed using an Agilent 7890A Network GC system coupled with 5975C VL MSD (Santa Clara, CA) with a DB-5MS capillary column (30m x 0.25 mm, 0.25 µm film thickness). The inlet temperature was set to 250°C and the interface temperature was set to 280°C. The source and quadrupoles were 230°C and 150°C, respectively. Each injection was 2 µL in split mode with a 5:1 split ratio. The initial oven temperature was held at 260°C for 0.5 min and ramped to 290°C at a rate of 30°C/min with a final hold of 17 min. The total run time was 18.5 minutes. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. Data was acquired using selective ion monitoring (SIM) for the following ions: 432, 273, and 94 m/z for Salvinorin A; 390, 273, and 94 m/z for Salvinorin B; and 291, 249, and 124 m/z for testosterone-D_3. Quantitation ions are underlined.

RESULTS AND DISCUSSION

Analytical recovery was evaluated using 30 ng/mL Salvinorin A/B in urine. Recovery of Salvinorin A was 82 ± 8 % (n=6) and 91 ± 4 % for Salvinorin B (n=6).

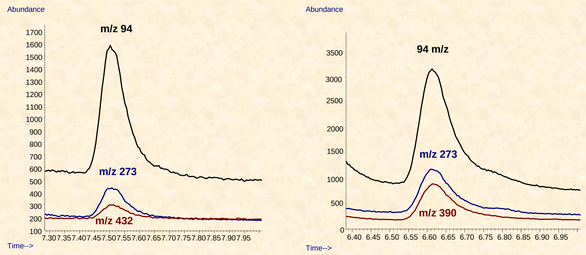
The calibration model was investigated using drug-free urine fortified with analyte of interest between 0 – 5,000 ng/mL. Replicate calibration data and analysis of residual plots were used to determine the most appropriate model. Non weighted linear regression was selected. Calibration models were linear between 0 – 1,000 ng/mL Salvinorin A/B (Figure 2). Calibrations were routinely performed between 0 to 500 ng/mL and no carryover was observed at 1,000 ng/mL.

Figure 2. Calibration Models



The limit of detection (LOD) was determined by fortifying drug into drug-free urine (from three sources) and analyzing in duplicate over five days. The acceptance criteria for reporting was a relative retention time within 2% of expected, ion ratios within 20% of expected and a signal to noise ratio of 3:1 or more. The limit of quantitation (LOQ) was determined in an analogous fashion, except that the criteria for acceptance included a signal to noise ratio of 10:1 or more and a calculated concentration within 20% of the expected concentration (in addition to the retention time and ion ratio requirements). The LOD and LOQ for Salvinorin A were 5 ng/mL and 10 ng/mL, respectively. The LOD and LOQ for Salvinorin B was 20 ng/mL. Extracted ion chromatograms (EICs) for Salvinorin A and B at the limit of quantitation are shown in Figure 3.

Figure 3. EICs for Salvinorin A/B at the LOQ



Precision and bias were evaluated at 50, 200 and 400 ng/mL over five days in triplicate at each concentration tested. Intra-assay precision and bias is summarized in Table 1. Data represents the range of precision and bias, which were within acceptable parameters (<20%). Table 2 summarizes the inter-assay precision and bias for all assays over five days (n=15).

Table 1. Inter-assay Precision and Bias

Concentration	Compound	CV (%)	Accuracy (%)	Bias (%)
50 ng/mL	**Salvinorin A**	4.3-13.1	84-106	-16 to 0
	Salvinorin B	4.3-13.4	84-106	-16 to 6
200 ng/mL	**Salvinorin A**	8.3-16.2	89-113	-11 to 13
	Salvinorin B	1.2-11.9	82-104	-18 to 4
400 ng/mL	**Salvinorin A**	1.8-11.4	88-106	-12 to 6
	Salvinorin B	1.0-18.2	82-103	-18 to 3

Table 2. Inter-Assay Precision and Bias

Concentration	Compound	Mean ± SD (ng/mL) (n=15)	CV (%)	Accuracy (%)	Bias (%)
50 ng/mL	**Salvinorin A**	48.7 ± 6.2	12.8	97	-3
	Salvinorin B	47.5 ± 7.5	15.7	95	-5
200 ng/mL	**Salvinorin A**	201.2 ± 29.0	14.4	101	1
	Salvinorin B	182.0 ± 20.5	11.2	91	-9
400 ng/mL	**Salvinorin A**	389.0 ± 36.6	9.4	97	-3
	Salvinorin B	378.0 ± 54.4	14.4	94	-6

Interferences caused by internal standard, common drugs (38), endogenous, and structurally similar compounds were evaluated in accordance with SWGTOX recommendations. No interferences were present. Retention times for the salvinorins and divinatorins are summarized in Table 3. Finally, dilution integrity was verified by performing a 1:10 dilution on a urine sample containing 2,000 ng/mL Salvinorin A/B in triplicate.

Table 3. Retention Times for Salvinorins and Divinatorins

Compound	MW	Retention time
Salvinorin A	432	7.9
Salvinorin B	390	6.7
Salvinorin C	474	9.0
Salvinorin D	432	9.6
Salvinorin E	432	8.4
Salvinorin F	374	7.9
Salvinorin G	430	5.6
Divinatorin A	332	2.8
Divinatorin B	362	4.4
Divinatorin C	374	5.5
Testosterone-D_3 (IS)	291	3.8

SUMMARY

A validated procedure for the identification of Salvinorin A and B from urine is presented. Despite the fact that the range of forensic interest is typically very low, an optimized extraction and GC/MS analysis proved highly effective. In addition to its use as a recreational drug, the anti-addiction, antidepressant and neuroprotective effects of Salvinorin A have drawn attention for clinical use in humans (Orton, 2014). This potential for use as a novel therapeutic agent highlights the importance of having validated methods of analysis in biological fluids.

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