

# The Detection and Quantitative Analysis of the Psychoactive Component of *Salvia divinorum*, Salvinorin A, in Human Biological Fluids Using Liquid Chromatography–Mass Spectrometry\*

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## Abstract

*Salvia divinorum*, a member of the mint plant family, has hallucinogenic properties that have become increasingly sought after by recreational drug users. The main psychoactive component, salvinorin A, has potency comparable to lysergic acid diethylamide. Though still legal to possess in most of the United States and much of Europe, little is known regarding the compound's long-term health effects, addiction liability, and pharmacokinetics. Limited data are available in the scientific literature, and few analytical methods are published for the detection in human biological fluids. These factors contribute to the unfamiliarity of the compound and complicate the method development process necessary to accommodate special requested testing for salvinorin A. A sensitive analytical method for the detection and quantitation of salvinorin A in human biological fluids was developed and validated to resolve analytical shortcomings. The method utilizes a solid-phase extraction technique coupled with liquid chromatography–electrospray ionization mass spectrometry operated in selected ion monitoring mode. The assay has a linear range of 5.0–100 ng/mL with a correlation coefficient of 0.997. The limit of detection and limit of quantitation were experimentally determined as 2.5 and 5.0 ng/mL, respectively. The method has been applied to blood and urine samples successfully and can be used to detect the presence of salvinorin A in forensic testing.

## Introduction

*Salvia* identifies a large group of plants containing almost 1000 species that belong in the mint family Lamiaceae (1). One particular species, *Salvia divinorum*, has been identified as

having hallucinogenic properties (1). The plant was first used by the Mazatec Indians in Oaxaca, Mexico in the 1800s and still plays a role in their healing and religious rituals (1). Recently, the literature reports use of the plant's recreational psychoactive effects by an increasing number of people in the United States and Europe (2,3).

Chemical analyses conducted on the leaves of *Salvia divinorum* identified the active component, salvinorin A, and a proposed mechanism for its hallucinogenic effects. Salvinorin A (Figure 1), discovered and isolated in 1982, has been identified as the only psychoactive component of the plant (4,5). Further research was conducted to determine which receptors salvinorin A targets (6). Unlike classic hallucinogens, such as lysergic acid diethylamide (LSD), salvinorin A does not show high affinity for the 5-hydroxytryptamine (5-HT serotonin) class of receptors, specifically the 5-HT(2A) receptor type (6). Instead, salvinorin A was identified as having a high affinity for the kappa opioid receptor, making it the only known non-nitrogenous opioid agonist (6).

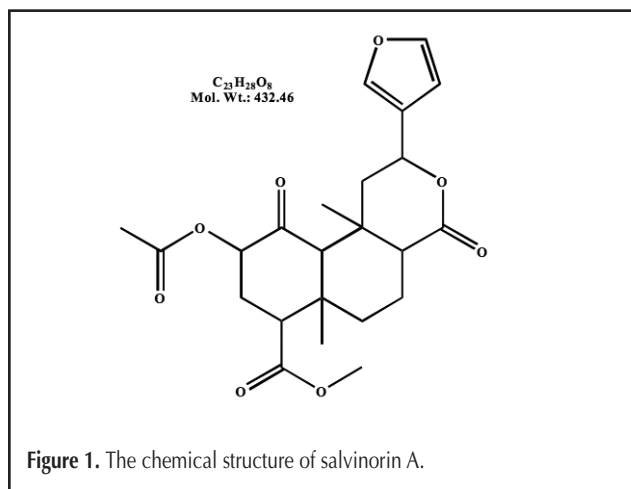


Figure 1. The chemical structure of salvinorin A.

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The plant and its psychoactive component, salvinorin A, are listed on the United States Drug Enforcement Administration's Drugs and Chemicals of Concern List; however, they are currently unscheduled substances (7,8). Bans of the plant and drug are being increasingly enforced at the state level. Louisiana, Delaware, Missouri, Tennessee, Oklahoma, Maine, and North Dakota have passed legislation prohibiting at least one of or both the plant and drug, and several other states have similar legislative bills pending (7). The popularity of the plant has increased internationally with a few countries (Australia, Belgium, Denmark, Estonia, Finland, Italy, Spain, and Sweden) declaring possession, use, or distribution illegal (8). Websites and head shops selling psychotropic plants, drug paraphernalia, and dietary supplements term *Salvia divinorum* a legal hallucinogen (9). Street names include diviner's sage, magic mint, and salvia (7).

The most common and effective route of administration is via the lungs by smoking dried leaves packed into a water bong or pipe (1). Orally, salvia leaves are chewed using a quid, and the juice is retained in the mouth to increase absorption through the mucosa (1). Teas are also prepared by steeping the leaves in hot water; however, this is a relatively ineffective method as salvinorin A is poorly absorbed in the gastrointestinal tract (1). Salvinorin A can also be extracted from plant leaves, and the material may be vaporized and inhaled (1,10). Once inhaled, the effects are felt within 60 s of exposure and last approximately 60 min (10). The required smoked dosage is 250–500 µg and is comparable to LSD, which is considered the most potent synthetic hallucinogen known to exist, with a dosage of 50–200 µg (1,6,11). Psilocybin and mescaline, other commonly abused hallucinogens, have much larger doses of 5 and 100 mg, respectively (11). This supports the contention that salvinorin A is the most potent naturally occurring hallucinogen isolated to date (6).

Although the overall experience of using salvinorin A has been described as unique, the effects are similar to other hallucinogenic drugs. Users describe depersonalization, out of body experiences, and uncontrollable laughter, which are common with other hallucinogens (1,11).

There are several published methods for the isolation of salvinorin A from plant material, but there are few analytical methods for the detection of the compound in human biological fluids (12). Attempts were made to duplicate previously published methods, but the results were unsatisfactory, and a new method was developed and validated. The following details a simple and robust analytical method for accurately measuring the concentration of salvinorin A in human blood and urine.

## Experimental

### Chemicals and reagents

All solvents were high-performance liquid chromatography grade and were purchased from Fisher Scientific (Pittsburgh, PA). Salvinorin A (> 98% purity) was purchased in crystalline form from ElSohly Laboratories (Oxford, MS). Warfarin

(> 98% purity) was purchased from Abbott Laboratories (Abbott Park, IL) and used as internal standard. Oasis® HLB (WAT094226) solid-phase extraction columns were obtained from Waters (Milford, MA).

### Standards, calibrators, and controls preparation

A stock standard of salvinorin A was prepared in acetone and stored at –80°C in an amber vial. The standard and serial dilutions were prepared every two weeks and kept at this extremely low temperature to prevent degradation. Acetone was used because the solid form of salvinorin A is insoluble in methanol, acetonitrile, and deionized water.

Deuterated salvinorin A is not commercially available; therefore, a different compound was chosen as the internal standard. Several factors were used to make this decision, including structural similarities, extraction performance, availability to the laboratory and most notably, the population of salvinorin A users. Warfarin, an anticoagulant, was chosen as the internal standard because it shares chemical structure properties and behaves similarly to salvinorin A during extraction. More importantly, its presence is highly unlikely in the case samples requesting testing for salvinorin A as the majority of samples will be collected from young, healthy, active-duty military members. The compound was also immediately available to the laboratory. A stock standard of warfarin was prepared in deionized water and stored at 5°C in an amber vial.

Calibrator and control samples were prepared in 1.0 mL of certified drug-free urine at concentrations of 5, 10, 25, 50, and 100 ng/mL. Three controls were prepared at 7.5, 40, and 75 ng/mL and were used throughout method validation. The calibrators and controls were spiked prior to each extraction.

### Sample preparation and extraction

Urine samples (1 mL) were prepared by the addition of 2 mL of 0.1 M acetate buffer (pH 4.3), 50 µL of concentrated phosphoric acid, and 10 ng/mL of internal standard (warfarin). Three-milliliter Waters Oasis® HLB columns were sequentially conditioned with 3 mL methanol, 3 mL deionized water, and 1 mL 0.1 M acetate buffer (pH 4.3). Samples were applied to the columns and allowed to pass through gravimetrically. The columns were washed with 3 mL deionized water and 2 mL 0.1 M acetic acid and dried for 15 min under 25 psi of nitrogen. A final wash step of 2 mL of 10% methanol in water was applied, and the columns were dried for an additional 15 min under 25 psi of nitrogen. Samples were eluted with 2 mL of 25% acetonitrile in dichloromethane, and the eluates were evaporated to dryness under nitrogen at room temperature. The residues were reconstituted with 50 µL of a 1:1 ratio of mobile phase (acetonitrile and 0.1% formic acid in water) and transferred to polypropylene autosampler vials for analysis (12,13).

### Instrumental analysis

The samples were analyzed for salvinorin A using an Agilent Technologies (Palo Alto, CA) 1100 liquid chromatography–mass spectrometry (LC–MS) SL system comprised of a vacuum degasser, binary pump, autosampler, and thermostatted column compartment. Five-microliter injections

were made onto a Luna® phenyl-hexyl column (Phenomenex, 100 mm × 2.1-mm i.d.,  $d_p = 3 \mu\text{m}$ ) at a temperature of 30°C. The gradient mobile phase consisted of 0.1% formic acid and acetonitrile at a flow rate of 0.300 mL/min. The initial 40% gradient of acetonitrile was held for 1 min, increasing to 75% over the next 8 min, then to 90% in the final 2 min with a post equilibration time of 5 min. The total run time was 16 min. An electrospray interface in positive mode was used with a nebulizer pressure of 35 psi and 9.0 L/min of nitrogen drying gas at 325°C. Flow injection analysis of salvinorin A and warfarin standards were conducted to determine optimal fragmentor voltages for quantitation purposes.

The single-quadrupole MS was operated in positive ion selective monitoring (SIM) mode. For quantitation,  $m/z$  433 ( $M^+$ ), 373, and 295 were monitored for salvinorin A, and  $m/z$  390 ( $M^+$ ) and 251 were monitored for warfarin. The molecular ions were used for quantitation. A summary of the selected ions and their corresponding fragmentor voltages are shown in Table I.

### Method validation

The method was validated using certified drug-free urine, and the calibrators and controls were spiked prior to each extraction. This was necessary because of the observed breakdown of salvinorin A over a short period of time. Method validation included the determination of the limits of detection (LOD) and quantitation (LOQ), linearity, inter- and intraday precision, accuracy, and extraction efficiency.

The LOD was determined as the lowest concentration of drug where the calculated value exceeds  $\pm 20\%$  of the theoretical concentration, but the peak is within  $\pm 2\%$  of the calibrators' retention time and 20% of the calibrators' ion ratios. The LOQ is the lowest concentration that meets the described requirements with a calculated value within  $\pm 20\%$  of the theoretical concentration.

Five-point calibration curves were used to determine the linearity over a concentration range of 5.0–100 ng/mL. The target compound and internal standard peak-area ratios were plotted against the concentration generating a representative regression analysis.

Intraday precision was performed in one extraction by analyzing five aliquots of three controls with different concentrations in the established linear range. Interday precision was determined by analyzing one aliquot of each control over 10

extractions. The mean of the quantitative results were calculated for each concentration. The coefficient of variance (CV) was also calculated, and it is defined as the percentage value of the standard deviation divided by the mean. This value must be less than 15% for the method to be considered validated per laboratory guidelines. The accuracy of the method was calculated as the percent difference between the theoretical concentration and the experimental concentration. This value must be less than 20% for method validation per laboratory guidelines.

The total extraction efficiency of the method was determined by comparing the abundance of an unextracted sample of salvinorin A with the abundance of an extracted urine sample. This measures the total amount of salvinorin A lost, including percent lost during the extraction and the loss due to ion suppression.

## Results

### Chromatography and method validation

A total ion chromatogram of a 25 ng/mL standard of salvinorin A is shown in Figure 2. Excellent peak shape and baseline resolution was obtained for the target compound and internal standard. The extracted SIM ions for a 7.5 ng/mL control sample of salvinorin A are shown in Figure 3.

The assay proved to be linear with a mean correlation coefficient ( $N = 10$ ) of 0.997 for salvinorin A. The LOD and LOQ were calculated to be 2.5 and 5.0 ng/mL, respectively. Three controls (7.5, 40, and 75 ng/mL) within the established linear range were used for the remaining method validation, and the results are summarized in Table II.

Interday precision yielded standard deviations less than 5 and %CVs less than 8.5% for the three controls. Intraday precision yielded standard deviations less than 6 and a %CV less than 7.5%. The method proved to be accurate as the percent differences between the theoretical value and the experimental value for the three controls were less than 6.5%. These results met criteria provided by an in-house method validation procedure.

The total extraction efficiency was determined at three con-

Compound	Ions $m/z$	Fragmentor Voltage
Salvinorin A	433*	120
	373	140
	295	275
Warfarin (internal standard)	390*	225
	251	140

\* Quantitation ion.

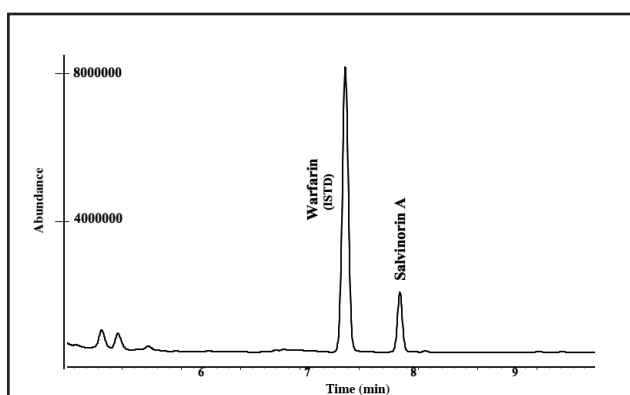


Figure 2. Total ion chromatogram of a 25 ng/mL urine extracted standard of salvinorin A.

centrations within the linear range. The total percent recovered was above 70%, which was deemed acceptable, and the ratio of percent recovered between salvinorin A and internal standard was consistent. This indicates salvinorin A and the internal standard behave similarly during extraction. More importantly, the ion suppression was minimal (< 30%) and affected both compounds equally, thus ensuring accurate quantitative results.

### Blood samples

Method validation was conducted using certified drug-free urine instead of certified drug-free blood for several reasons. The half life of salvinorin A is relatively short, and this, in conjunction with the low dosage of the drug, mandates that a blood sample would have to be collected almost immediately after exposure to detect the compound. Researchers of a patient study planned to analyze blood samples from two people exposed to salvinorin A but were unsuccessful (14). The two patients were too impaired to be approached with needles without potentially endangering themselves or the laboratory technicians collecting the samples.

Parameter	Value
Linearity* ( $R^2$ )	5.0–100 ng/mL 0.997
Limit of Detection	2.5 ng/mL
Limit of Quantitation	5.0 ng/mL
Extraction Efficiency	71.7%
Precision	
Interday <sup>†</sup>	CV < 8.5%
Intraday <sup>‡</sup>	CV < 7.5%
Accuracy	
Interday <sup>†</sup>	0.3–6.2%
Intraday <sup>‡</sup>	4.5–9.5%

\* Five-point calibration curve, 10 extractions.  
<sup>†</sup> One aliquot of 3 different concentrations, 10 extractions.  
<sup>‡</sup> Five aliquots of 3 different concentrations, 1 extraction.

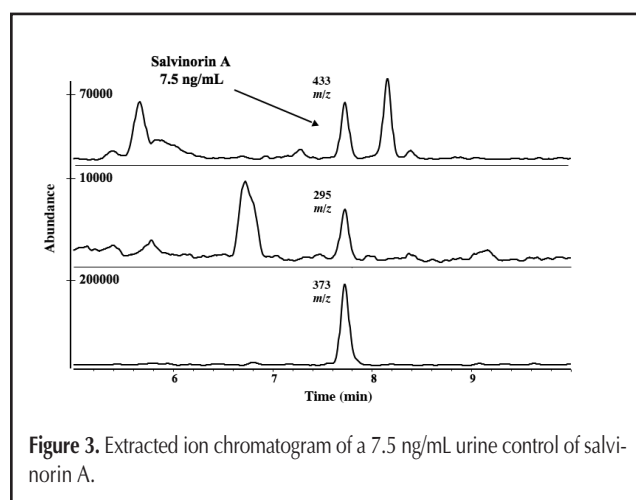


Figure 3. Extracted ion chromatogram of a 7.5 ng/mL urine control of salvinorin A.

After method validation was completed, a unique case was submitted for salvinorin A testing and only blood was available for analysis. The method was conducted using a blood calibration curve and controls and yielded acceptable results, including a linear calibration curve and quality controls within established quantitative ranges.

### Discussion

Reliable data concerning salvinorin A's distribution, elimination, metabolism, and toxicity are limited. The relative obscurity of the drug, lack of patient studies, and few analytical detection methods using human biological fluids are a few reasons for the limited details regarding its biotransformation. In 2005, a study was published in which two people smoked salvinorin A and their saliva and urine were analyzed using gas chromatography–mass spectrometry (GC–MS) (14). Low levels of salvinorin A were detected in urine (11.1 and 2.4 ng/mL) after 9.5 h post administration (14). These results demonstrate the need for a sensitive analytical detection assay to confirm use of the drug.

Further research is required to determine metabolic pathways and primary metabolite(s). In 2005, a quantitative method was published utilizing LC–MS with an atmospheric pressure chemical ionization interface (12). Biological fluids from primates treated with salvinorin A were analyzed (12). The parent compound was detected, in addition to a structurally related compound the authors identified as salvinorin B, a possible metabolite. The analytical results illustrated an inversely proportional relationship between the concentrations for salvinorin A and salvinorin B. This may be attributed to salvinorin A metabolizing into salvinorin B. These results, however, have yet to be corroborated by other research groups (12). Due to the low concentrations of the parent compound in biological fluids, identification of a specific, primary metabolite at higher concentrations may simplify detection and be used as an alternative marker to identify salvinorin A use.

### Conclusions

A simple solid-phase extraction method using LC–MS instrumentation was developed to detect and quantitate the psychoactive compound of *Salvia divinorum*, salvinorin A, in human biological fluids. Several methods are available to extract salvinorin A from plant material, but few are reported to detect the compound in human biological fluids. This method allows for the accurate measurement of salvinorin A in human blood and urine samples and is practical for use in the forensic laboratory setting. The method was validated and the results proved the method to be reliable and accurate. Continued research is required to provide insight into the biotransformation of salvinorin A, especially to determine its primary metabolite.

## Acknowledgments

This work was funded in part by the American Registry of Pathology, Washington, D.C. 20306-6000.

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Manuscript received February 14, 2008;  
revision received April 22, 2008.