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Determination of Salvinorin A in body fluids by high performance liquid chromatography–atmospheric pressure chemical ionization

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Abstract

Salvinorin A was quantitated in human and rhesus monkey plasma, rhesus monkey cerebrospinal fluid, and human urine by negative ion LC–MS/APCI. The method for Salvinorin A has been fully validated, the LLOQ using FDA guidelines is 2 ng/mL for 0.5 mL plasma samples. The linear range was from 2 to 1000 ng/mL. Several derivatives in the Salvinorin family can also be analyzed by this method; d_3-Salvinorin A was prepared and used as internal standard. The metabolite Salvinorin B can be semi quantitatively determined. The method has been used to establish that Salvinorin B is the principal metabolite of Salvinorin A ex vivo and to establish the analytical method to study in vivo samples.

Keywords: Salvinorin; LC–MS; APCT; Analysis; Urine; Plasma; Body fluids; Metabolism; Salvinorin A

1. Introduction

The Mexican mint, *Salvia divinorum*, is a hallucinogenic plant whose availability has recently increased rapidly, partially due to internet trading [1]. Among the structurally related compounds found in the plant are Salvinorin A, **1**, and Salvinorin B, **2** (Fig. 1). At present, only **1** appears to be pharmacologically active. Currently the plant is unregulated and legally available in all countries except Denmark, Italy, and Australia. The leaves of the plant and various extracts and tinctures are readily available over the internet [2]. The active ingredient, Salvinorin A, is typically self-administered in humans either by smoking or buccal absorption [3,4]. Salvinorin A based products can produce profound hallucinations lasting up to 1 hour [3,5]. It is expected that misuse of *Salvia divinorum* based products will increase rapidly [1].

Rather than affecting the serotonin 5-HT_{2A} receptors as do classical hallucinogens, **1** is a potent, efficacious, and selective κ opioid receptor agonist [6–8]. Selective synthetic κ opioid receptor agonists have been explored as analgesics with potential for reduced dependence and tolerance. However, dysphoria, diuresis, and psychotomimesis are associated with their administration in clinical studies in humans [9,10]. A growing body of evidence indicates that κ receptors may be involved in the modulation of some abuse related effects of CNS stimulants [11–14].

κ Agonists are able to modulate the behavioral and neurochemical effects of cocaine [11]. They appear to attenuate cocaine's discriminative stimulus properties, its conditioned reinforcing effects, and its self-administration. κ Agonists also lowered the reinstatement of cocaine-reinforced responding. The findings indicate the endogenous κ opioid receptor/dynorphin system may be involved in the physiological modulation of some abuse related effects of cocaine, offering a valuable pharmacological target to treat cocaine abuse or its relapse. However, while κ opioid receptor agonists are effective in reducing cocaine self-administration in monkeys, they produce side effects including sedation and vomiting [11]. It has been speculated

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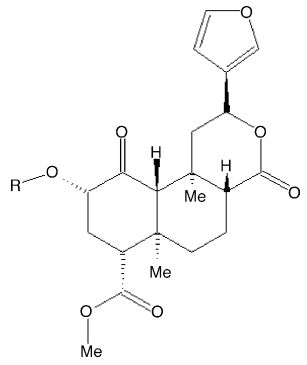

Fig. 1. Salvinorin. R = **1**, $COCH_3$; **1A**, $COCD_3$; **2**, H.

that the addition of μ agonist/antagonist activity to the κ agonist might lessen the incidence of side effects and encompass a useful treatment for cocaine abuse [11].

A method for determining **1** in plant tissues by HPLC with UV detection exists [15]. The presently reported method is 3 orders of magnitude more sensitive than the previously reported method. At present, there are no methods available for the detection of **1** and related analogues in biological fluids. In addition, the identities of metabolites of **1** are unclear. In order to support the study of the disposition of **1** in vivo, we set out to develop a method to determine the concentration of **1** in biological fluids.

2. Experimental

2.1. Chemicals and solutions

Salvinorin A and B were extracted from *Salvia divinorum* as previously described with modifications [16–19]. d_3-Salvinorin A, **1A**, was prepared from Salvinorin A [19]. Purities of the above were >98%. Salvinorin A for use in monkey studies was commercially obtained from Biosearch (Novato, CA, USA) and was approximately 92% pure, the residual was mostly Salvinorin B. ACS grade ammonium hydroxide, dimethylsulfoxide, dichloromethane, and Optima HPLC grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Ammonium acetate (AR grade) was purchased from Mallinckrodt Baker Inc. (Paris, KY). HPLC grade water was from a MilliQ system (Millipore Corp., Bedford, MA). Human plasma was obtained from the DeGowin Blood Center at University of Iowa Hospitals.

2.2. Blank plasma, plasma standards, and patient samples

To prepare plasma standards, **1** was dissolved in dimethylsulfoxide (DMSO) to make a 1 mg/mL solution. Salvinorin B was dissolved in acetonitrile to make a 0.5 mg/mL solution. These two solutions were combined and diluted with water to form a concentrated working standard of 10 μg/mL. This standard was serially diluted with water to 1 and 0.1 μg/mL to form less concentrated working standards. Calibration standards were prepared by adding between 10 and 100 μL of the appropriate working standard to sufficient human plasma to make 0.5 mL in a 2 mL tube. The standard curve consisted of samples containing 2, 10, 20, 50, 100, 200, and 1000 ng/mL. Internal standard solution was prepared by dissolving **1A** in DMSO to 2 mg/mL and serially diluting with water to 2 μg/mL. Water (500 μL), phosphoric acid (50 μL) and internal standard (**1A**, 50 μL) were added to each sample. Blanks, null blanks, controls, monkey plasma, and monkey cerebrospinal fluid controls were prepared in the same manner. All solutions were mixed and centrifuged 2 min at 16.1 × *g*. The above samples were subjected to solid phase extraction prior to instrumental analysis.

2.3. Blank urine and urine standards

To prepare urine samples, appropriate amounts of Salvinorin working standards were added to sufficient urine to make 1 mL in a 2 mL tube. Phosphoric acid (50 μL) and internal standard (50 μL) were added. The standard concentrations were 1, 5, 10, 25, 50, 100, 250, and 500 ng/mL of **1,2** with 100 ng/mL of **1A**. Blanks and null blanks were prepared in the same manner. All solutions were mixed and centrifuged 2 min at 16.1 × *g*. Control samples at 8 and 40 ng/mL were also prepared to make 0.5 mL spiked urine. The samples were subjected to solid phase extraction prior to instrumental analysis.

2.4. Solid phase extraction

Oasis HLB (30 mg, 1 mL) solid phase extraction cartridges (Waters Corporation, Milford, MA) were used for sample preparation. A Cerex SPE processor from Varian (Palo Alto, CA) using nitrogen to modulate flow was used. All flow rates were approximately 1 mL/min. The cartridges were conditioned with 1 mL methanol followed by 1 mL water. After loading the samples, the cartridges were washed with 1 mL of 10% methanol/water and dried with nitrogen for 5 min. The analytes were eluted from the cartridge with 1 mL of 75% dichloromethane in acetonitrile. The eluate was collected in 13 mm × 100 mm glass tubes and solvent removed under flowing nitrogen at 35 °C. The residue was reconstituted in 100 μL of 75% acetonitrile/water and transferred to an autosampler vial for analysis.

2.5. Instrumentation

The instrumentation system consisted of a Shimadzu 2010A LC–MS platform in APCI negative mode operating under LCMSSolution software (Version 2.04H3, Shimadzu, Columbia, MD, US). The analytical column was a Phenomenex Polar-RP (4 μm, 80 Å, 2.0 mm × 150 mm; Phenomenex Corp., Torrance, CA, US) preceded by a Phenomenex Polar-RP SecurityGuard guard (2.0 mm × 4 mm) column. Separation conditions were: sample temperature,

15 °C; column temperature, 23 °C (±3); sample injection volume, 15 μL. The analysis was isocratic at 0.25 mL/min flow. Solvent A (50%) was 4 mM ammonium acetate adjusted to pH 7.2 ± 0.2; solvent B (50%) was acetonitrile. The total run time for a LC–MS analysis was 9 min.

The mass spectrometer was tuned using a polyethylene glycol solution following the manufacturer's protocol. The scan interval was 0.3 s, microscan 0.1 amu, APCI temperature was 350 °C, the CDL temperature 250 °C, and the block temperature 200 °C. Nitrogen flows: APCI, 2.5 L/min; drying gas, 0.02 MPa.

2.6. Rhesus monkey studies

All studies in animals were reviewed by the Institutional Animal Care and Use Committee of the Rockefeller University, and were in accordance with Guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Health Council (Department of Health, Education and Welfare, Publication ISBN 0-309-05377-3, revised 1996). An ex vivo study of the metabolism of **1** was conducted. Fresh whole blood was taken from an adult, gonadally intact rhesus monkey and held at 37 °C. The blood was spiked with **1** to a concentration of 1900 ng/mL. Six samples were taken over the course of 60 min, cooled on ice and plasma separated. Samples were stored at −78 °C until extraction. Volume limitations necessitated 200 μL samples for extraction. A baseline sample prior to spiking was also collected. Cerebrospinal fluid was withdrawn from the cisterna magna, by percutaneous puncture, after administration of ketamine (10 mg/kg, i.m.) from a monkey never exposed to **1**.

3. Results and discussion

The Salvinorin concentrations were calculated from the peak-area ratio of **1** or **2** to **1A** for standards and samples. Peak areas of standards taken through solid phase extraction were approximately 102% those of standards evaporated and reconstituted. The linear least squares equations were calculated with $1/C$ weighting. For **1**, the mean equation of the linear portion of the curve derived from human plasma was $0.0145x + 0.0264$ (R.S.D. slope 5.4%, R.S.D. intercept 9.1%), the correlation coefficient was 0.999; the linear range was from 2 to 1000 ng/mL, based on a 0.500 mL sample. For Salvinorin B, the equation of the linear portion of the curve derived from human plasma was $0.00547x + 0.0168$; the correlation coefficient was 0.985, and the linear range was from 50 to 1000 ng/mL, based on a 0.500 mL sample. Series of plasma controls (0.5 mL) spiked with **1** at 8 and 40 ng/mL were analyzed for accuracy and precision (Table 1). The LLOQ of 2 ng/mL was determined according to FDA guidelines as a control value where the R.S.D. is <20% and analyte signal is >5 times the blank matrix. Salvinorin B does not ionize as well and in plasma matrix interferences are not completely removed. Samples below 50 ng/mL show considerable variability in their results. Salvinorin B is not believed to be biologically active and its presence in in vivo samples could not be clearly determined (unpublished observations). At present, there is no compelling reason to fully develop a trace quantitative analysis for Salvinorin B; we classify the analysis of Salvinorin B as semiquantitative, for use in monitoring ex vivo studies where the compound is formed in larger quantities.

Table 1
Accuracy and precision data for **1**

Concentration (ng/mL)	Accuracy (%)	R.S.D. (%)
Intraday ($n = 10$)		
2	104.0	11.2
8	96.4	4.0
40	100.4	3.1
Interday (3/day × 5 days)		
8	98.7	10.3
40	101.1	4.3

Recently published work found parent related ions $[M + 23]$ could be found for **1** and several derivatives using electrospray ionization (ESI) in positive mode for high resolution mass spectroscopy [8]. The authors purpose was only to identify a series of synthesized derivatives. In our hands positive or negative mode ionization using ESI gave multiple ions; the parent ion and easily assigned adducts were relatively small. Positive ion mode APCI yielded one major fragmentation ion along with several minor fragmentation ions. Very little $[M + 1]$ ion was observed. The fragmentation ions observed had cleaved away the functional group we were most interested in observing and altering. Negative ion conditions using APCI produced $[M - 1]$ ions in high yield. As ion intensities were also somewhat higher in negative ion mode, we opted for negative mode in order to obtain parent masses and to be able to use the deuterated compound as internal standard.

There are no published data on the absorption, distribution, metabolism or excretion of **1**. To better support future work, we conducted herein a preliminary examination of ex vivo spiked human urine and ex vivo spiked rhesus monkey cerebrospinal fluid and blood to determine if the extraction method used was applicable without modification to these

Table 2
Alternative matrix results calculated against human plasma calibration curve

Spiked concentration (ng/mL)	Recovered (ng/mL)	% Recovery
Human urine		
8	8.5	106
40	41.7	104
Rhesus monkey cerebrospinal fluid		
20	22.8	114
100	102	102
Rhesus monkey blank plasma		
8	7.9	99.8
40	39.2	98.0

Table 3
Recovery of 50 ng **1** from different plasma volumes

Volume of spiked plasma (mL)	Found (ng)
0.200	51
0.500	48
1.000	50

biological matrices. To this end, samples were spiked, extracted, and calculated using the plasma calibration curve (Table 2). Residual matrix peaks were absent in either of these matrixes, while present in plasma. We also evaluated possible effects of sample volume by spiking 50 ng (50 μL of 1 μg/mL) of **1** into 150, 450, and 950 μL of plasma (Table 3). As generally expected, recovery of analyte by SPE was unaffected by sample volume within this range. A calibration curve was also constructed using urine to determine linearity over the full range found for plasma. The parameters of the straight line found were within the range established for curves constructed from spiked plasma.

Fig. 2 illustrates ion chromatograms of spiked and extracted human plasma, blank monkey plasma, and monkey plasma from blood incubated with **1** for 15 min at 37 °C. The assay was used to obtain preliminary results for the disappearance of **1** and formation of Salvinorin B in incubated monkey blood. The concentration–time curve for **1** and **2** in incubated monkey blood is shown in Fig. 3. Salvinorin B was seen to increase steadily over the time course as **1** decreased. When **1** is heated in water at 37 °C for 45 min, the Salvinorin A peak area was constant and the Salvinorin A to Salvinorin B peak area ratio is unchanged. Presumably, blood esterases convert Salvinorin A to Salvinorin B.

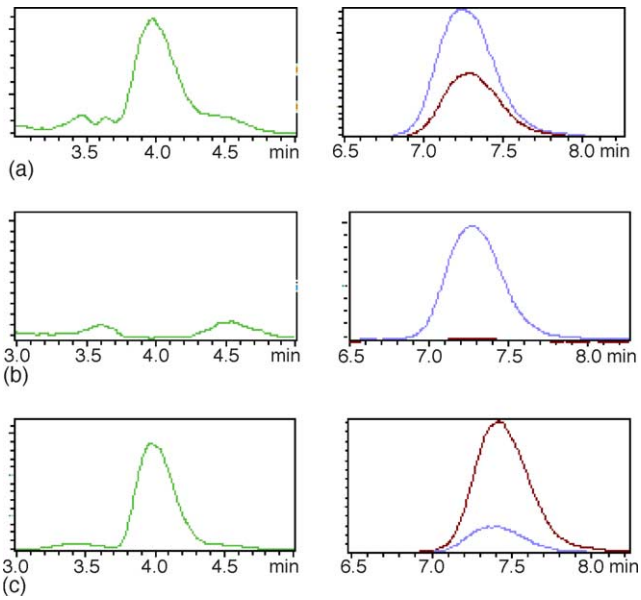

Fig. 2. Ion chromatograms of Salvinorin compounds. (a) A 100 ng/mL spiked human plasma, (b) monkey baseline blank, (c) monkey plasma from blood incubated ex vivo for 15 min. Left hand chromatogram is Salvinorin B, 4.0 min; right hand chromatogram is Salvinorin A and deuterated internal standard, 7.25 min (IS top ion trace).

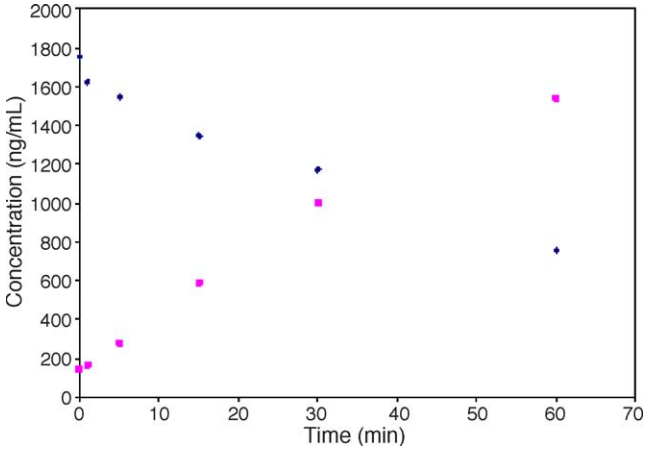

Fig. 3. Concentration time plot of Salvinorins in ex vivo monkey blood. (♦) Salvinorin A; (■) Salvinorin B.

4. Conclusion

A sensitive method for the determination of Salvinorin compounds in body fluids has been developed and its utility demonstrated in an ex vivo pharmacokinetic study supporting the feasibility of future in vivo investigations. This is the first study to demonstrate the formation of Salvinorin B as a metabolite of Salvinorin A in biological fluids. The ex vivo data suggests that Salvinorin A is metabolized by blood esterases primarily to Salvinorin B.

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