Determination of salvinorin A and salvinorin B in Salvia divinorum-related products circulated in Japan

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1. Introduction

Salvia divinorum (Lamiaceae), which is native to Oaxaca in Mexico, has been used for traditional medicine and spiritual practices because of its hallucinogenic properties[1]. In recent years, this plant has been widely used as a herbal dietary supplement or a herbal hallucinogen due to internet trading[2]. Singh has reported a psychiatric emergency case with paranoia, dejà vu, and blunted affect due to drug’s abuse[3]. This plant has been circulated as not only whole or pulverized leaf but also as “concentrated extract” products. The buying and selling of S. divinorum and its active ingredients have been controlled in several countries, including Japan[4].

Several neoclerodane diterpenoids (salvinorins A–I, divinatorins A–E, and salvidivins A–D) were isolated from S. divinorum[5–11]. In these diterpenoids, salvinorin A (SalA, Fig. 1) is mainly involved in the psychiatric activity. This compound is a potent and selective κ-opioid receptor agonist in vitro[12]. Salvinorin B (SalB, Fig. 1), a deacetylated form of SalA, is another important constituent, despite the deficiency of obviously pharmacological activity, because this compound is presumed to be a major metabolite of SalA in rhesus monkey blood[13].

There have been several reports of chemical analysis of SalA in S. divinorum and its related products. For example, Siebert analyzed the chloroform extract of the fresh leaf by thin layer chromatography[14]. In the method of Giroud et al., the ground fresh leaf in saturated ammonium buffer (pH 9.5) was extracted by chloroform–2-propanol (9:1, v/v), and the extract was analyzed by gas chromatography–mass spectrometry (GC–MS) after acetyl-derivation[15]. The same authors analyzed methanol extract of the dried leaf by GC–MS without derivatization[15]. Gruber et al.[16] and Wolowich et al.[17] analyzed the respective chloroform extracts of the lyophilized leaf and the “concentrated extract” product by high-performance liquid chromatography (HPLC) with UV detection. Medana et al. extracted the leaf by acetonitrile–water (1:1, v/v), and the extract was analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS)[4].

The extraction procedures shown in the aforementioned References ([14–17,4]) were not fully examined. For example, selection of extraction solvent, extraction efficiency, and how to remove pigments were not examined. Moreover, SalA concentrations in the “concentrated extract” products have been reported in only one study[17].

The aims of this study are (i) to examine the analytical method of SalA and SalB in the S. divinorum-related products and (ii) to analyze these products circulated in Japan. The present method...
2. Experimental

2.1. Samples and chemicals

Twelve samples (two dried whole-leaf products, one dried pulverized-leaf product, and nine “concentrated extract” products) sold as *S. divinorum*-related products were used in this study. These samples were obtained via the internet in Japan. As for the dried leaf products, their morphologic features observed under a scanning electron microscope were almost fully in accordance with the description in Ref. [14]. On the package of the “concentrated extract” product, its “concentration ratio” was described in the range between 2 to 25×. This ratio was afterward called as “labeled potency”.

Garden species of Salvia were purchased at gardening shops in Japan. These species used in this study were as follows: *Salvia elegans* “Scarlet Pineapple”, *Salvia forskohlii*, *Salvia ‘Indigo Spires’*, *Salvia glutinosa*, *Salvia guaranitica*, *Salvia blepharophylla*, *Salvia mexicana* “Limelight”, *Salvia buchananii*, *Salvia splendens*, and *Salvia farinacea*.

SalA was isolated from *S. divinorum* leaves as previously described with several modifications [18]. SalB was prepared from SalA according to a previously reported method [18]. These two compounds were identified by LC–MS/MS and quantified by a standard addition method. The spiked amounts were 0, 5, 10, and 20 ng/20 μL of the injection solution, which correspond to 0, 0.05, 0.1, 0.2, and 0.5 μg/mg sample (in the case of no dilution).

2.2. Standard solutions

Standard solutions were prepared regarding the purities of SalA and SalB as 100%. SalA (1 mg/mL) and SalB (0.25 mg/mL) solutions in acetonitrile were prepared as stock solutions. Their working standard solutions were prepared from the stock solutions by sequential dilution with acetonitrile. Concentrations of the working standard solutions were as follows: 5–200 μg/mL for SalA and 0.5–50 μg/mL for SalB. These solutions were stored at 4°C.

2.3. Extraction procedure

The whole-leaf samples were ground to a fine powder in a mortar before extraction. The other samples (a dried pulverized-leaf product and “concentrated extract” products) were supplied for extraction without pulverization.

Two milliliters of acetonitrile was added to 50 mg of the sample in a test tube, followed by shaking for 1 min and ultrasonication for 5 min. After centrifugation at 3000 rpm for 3 min, the supernatant was transferred to a volumetric flask. The residue was extracted once more with 2 mL of acetonitrile, shaken, ultrasonicated, centrifuged, and transferred in the same way. The combined extracts were made up to 5 mL with acetonitrile. In addition, most of the combined extracts originated from the “concentrated extract” products were appropriately diluted by acetonitrile. This dilution was carried out by that 1 mL of the combined extracts was transferred into another test tube, then 1, 3, or 9 mL of acetonitrile was added into this test tube and mixed well (respective dilution factor: 2, 4, and 10). The dilution factor selected for the each extract was the minimum necessary one to decolor it by the after-mentioned GCP treatment.

One hundred and sixty milligrams of GCP were added to 1 mL of these extracts (after dilution if necessary), followed by shaking for 1 min and centrifugation at 3000 rpm for 1 min. The upper layer was transferred to a separate vial. An equivalent volume of distilled water was added to this extract, then filtered through a 0.45-μm membrane (UNIFILTER, Whatman, Clifton, NJ, USA), and a 20 μL aliquot was used for the confirmation and quantitative analysis.

2.4. Quantitative analysis

SalA was quantified by HPLC with UV detection (210 nm) by an external standard method. A calibration curve was constructed by injection of working standard solutions diluted by an equivalent volume of distilled water. The injection amounts were 50, 100, 200, 500, 1000, and 2000 ng, which correspond to 0.5, 1, 2, 5, 10, and 20 μg/mg sample (in the case of no dilution). SalB was quantified by LC–MS in SIM mode at m/z 389 by a standard addition method. The spiked amounts were 0, 5, 10, 20, and 50 ng/20 μL of the injection solution, which correspond to 0, 0.05, 0.1, 0.2, and 0.5 μg/mg sample (in the case of no dilution).

2.5. Chromatographic and mass spectrometric conditions

Chromatographic separation was performed with a Mightysil RP-18 column (2.0 mm × 150 mm, 5 μm, Kanto Chemical, Tokyo, Japan) maintained at 40°C. The mobile phase was 0.05% formic acid in water and acetonitrile with a constant flow rate of 0.2 mL/min. The acetonitrile percentages were 0–1 min, 40%; 1–16 min, linearly from 40% to 70%; 16–18 min, linearly from 70% to 100%; 18–21 min, 100%; 21–35 min (re-equilibration step), 40%.

The confirmation analysis was carried out using a Thermo Fisher Scientific Accela series HPLC system (San Jose, CA, USA) and a Thermo Fisher Scientific LCQ Fleet ion trap mass spectrometer with an electrospray ionization (ESI) interface in the positive mode. MS data were collected in product ion scan mode. The precursor ions, collision energy, and scan ranges were m/z 373, 25%, m/z 313, 314, and 355 for SalA and m/z 373, 355, and 259 for SalB. The main mass conditions were spray voltage (5 kV), capillary voltage (50 V), capillary temperature (250°C), sheath gas flow rate (60 arb), auxiliary gas flow rate (10 arb), and sweep gas flow rate (10 arb).

Quantitative analysis was carried out using a Waters 2690 series HPLC system (Milford, MA, USA) equipped with a Waters 2996 diode array detector and a Waters ZQ single-quadrupole mass spectrometer with an ESI interface in the negative mode. MS data were collected in SIM mode at m/z 389. The main mass conditions were cone voltage (60 V), capillary voltage (4500 V), cone gas flow (50 L/h), desolvation gas flow (350 L/h), and desolvation temperature (300°C).

3. Results and discussion

3.1. Selection of an extraction solvent

Selection of an extraction solvent was based not only on extraction efficiency but also on cleanliness of the extract, because pigments such as chlorophylls extracted with the SalA/SalB would cause pollution of an HPLC column and an MS system. In the extraction solvents used in the previous reports, methanol [15], acetone [18], and acetonitrile/water (1:1, v/v) [4] were directly injectable solvents to a reverse-phase HPLC system. However, not
these solvents but acetonitrile was selected as an extraction solvent for the following reasons:

(i) Acetonitrile gave a clearer extract than these solvents.
(ii) Extraction efficiency of acetonitrile was approximately equivalent to those of these solvents.

3.2. HPLC analysis of SalA and SalB

3.2.1. Confirmation analysis

Fig. 2(A and B) shows typical MS/MS chromatograms and the MS/MS spectra obtained from a S. divinorum sample (sample #1, dried S. divinorum leaf product). The MS/MS spectra obtained from peaks eluted at 9.7 min and 5.4 min matched with those of SalA and SalB, thus confirming the presence of SalA and SalB.

3.2.2. Quantitative analysis

Quantification of SalA was performed by UV detection, as the sensitivity of UV detection is more stable than that of MS detection because the latter may suffer from phenomena such as ion suppression and contamination of the sample cone, causing a deterioration of sensitivity. Although UV detection is usually less sensitive than MS, the SalA levels in the samples were sufficient to be determined by UV detection. In contrast, SalB was quantified by LC–MS in SIM mode because the SalB content in the samples was insufficient to be detected by UV detection in a preliminary study.

Fig. 2(C and D) shows typical UV and SIM chromatograms obtained from a S. divinorum sample (sample #1, dried S. divinorum leaf product), respectively. The peaks for SalA and SalB had retention times of 10.5 min and 5.2 min, respectively. These peaks were not detected in the extract of the garden species of Salvia.

3.3. Extraction efficiency of SalA/SalB

In a preliminary study, SalA/SalB in the samples was extracted four times with acetonitrile to investigate the extraction efficiency. Nearly all SalA and SalB were recovered from the samples by two extractions with acetonitrile. Hereafter, two-time extraction was used for the subsequent analysis of SalA/SalB.

3.4. Removing pigments by GCP treatment

Pigments such as chlorophylls in the extract may interfere with the chromatographic analysis. GCP is often used to remove the pigments in the field of residual pesticide analysis. Decoloring effects and loss of SalA/SalB by GCP treatment were investigated.

Adding 160 mg of GCP to 1 mL of the extract was sufficient to remove the pigments in the extract originated from the S. divinorum leaf samples without dilution. However, this GCP amount was insufficient for decoloring the extract originating from most of the “concentrated extract” products. In such cases, the extract was successfully decolored by GCP treatment after the appropriate dilution (maximum dilution factor: 10) with acetonitrile.

The recoveries of SalA/SalB during GCP treatment were investigated by adding 160 mg of GCP to 1 mL of their solution.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-assay (n = 5)</th>
<th>Inter-assay (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.8 ± 0.1</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>3.0 ± 0.1</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>C</td>
<td>9.5 ± 0.8</td>
<td>11.1 ± 1.4</td>
</tr>
</tbody>
</table>

Each data represents mean ± S.D.
The SalA and SalB recoveries were 99.0 ± 1.2% and 96.5 ± 0.8% (mean ± S.D., 50 μg/mL each in acetonitrile, n = 5), respectively. This result indicated that the loss by GCP treatment was negligible. The stability of SalA/SalB in the acetonitrile extract in a refrigerator (at 4 °C, without light shielding) was evaluated using the extracts originating from a S. divinorum dry leaf product. Fig. 3 illustrates the time course of the residual ratios of SalA/SalB. There was obviously degradation of SalA/SalB in the non-GCP-treated extracts. In contrast, this degradation was not observed in the GCP-treated extracts. This finding suggested that any pigments remaining in the extracts originating from a S. divinorum dry leaf product. If the labeled potencies are adequate, it is expected that the SalA/SalB contents in the “concentrated extract” products are 4.1–38.9/0.26–2.4. These concentrations tended to increase in proportion to the labeled potency. Wolowich et al. have reported that the SalA/SalB contents in four “concentrated extract” products (labeled potency: 5 × 20 ×) were 0.126–0.951 μg/mg [17]. SalA concentrations in our samples were obviously higher than the reported values.

To evaluate adequacy of the labeled potencies in the “concentrated extract” products, SalA contents in them were standardized by their labeled potencies. This standardization was carried out by dividing the SalA contents by the labeled potencies. If the labeled potencies are adequate, it is expected that the standardized SalA contents are almost equal to those of the dried leaf products. However, the standardized SalA contents were significantly lower than those of the dried leaf products (p = 0.046, two-tailed unpaired t-test). This result indicates that their labeled potencies were lower than the expected ones.

### Table 2
SalA/SalB contents in the S. divinorum-related products

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>aLabeled potency</th>
<th>bDilution factor</th>
<th>Content (μg/mg)</th>
<th>cStandardized SalA content (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SalA</td>
<td>SalB</td>
</tr>
<tr>
<td>Dried leaf products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1×</td>
<td>1</td>
<td>5.0</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>1×</td>
<td>1</td>
<td>3.2</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>1×</td>
<td>1</td>
<td>3.3</td>
<td>0.17</td>
</tr>
<tr>
<td>“Concentrated extract” products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2×</td>
<td>2</td>
<td>4.1</td>
<td>0.27</td>
</tr>
<tr>
<td>5</td>
<td>7×</td>
<td>2</td>
<td>6.6</td>
<td>0.26</td>
</tr>
<tr>
<td>6</td>
<td>10×</td>
<td>1</td>
<td>11.7</td>
<td>0.32</td>
</tr>
<tr>
<td>7</td>
<td>10×</td>
<td>2</td>
<td>10.0</td>
<td>0.57</td>
</tr>
<tr>
<td>8</td>
<td>10×</td>
<td>2</td>
<td>12.7</td>
<td>0.77</td>
</tr>
<tr>
<td>9</td>
<td>14×</td>
<td>4</td>
<td>15.5</td>
<td>0.64</td>
</tr>
<tr>
<td>10</td>
<td>20×</td>
<td>4</td>
<td>27.3</td>
<td>1.8</td>
</tr>
<tr>
<td>11</td>
<td>20×</td>
<td>4</td>
<td>20.5</td>
<td>1.3</td>
</tr>
<tr>
<td>12</td>
<td>25×</td>
<td>10</td>
<td>38.9</td>
<td>2.4</td>
</tr>
</tbody>
</table>

### Notes

a Labeled potency of the dried leaf products was expressed as 1×.
b Dilution factor 1 means no dilution.
c Standardized SalA content (μg/mg): SalA content (μg/mg)/labeled potency.

Table 2 summarizes the labeled potencies, the dilution factors before the GCP treatment, and the SalA/SalB contents in the S. divinorum-related products. The SalA/SalB contents (μg/mg) in the three dried leaf products were 3.2–5.0/0.10–0.17 μg/mg. The reported contents in dried leaf samples were 7.6–7.8 μg/mg [4] and 0.89–3.70 μg/mg [16] for SalA, as well as 4.2–10.4 μg/mg for SalB [4]. The SalA contents in our samples were roughly in accordance with these reported values. In contrast, the SalB contents in our samples were obviously lower than the reported values. Unfortunately, the cause of this difference was unclear. The SalA/SalB contents (μg/mg) in the “concentrated extract” products were 4.1–38.9/0.26–2.4. These concentrations tended to increase in proportion to the labeled potency. Wolowich et al. have reported that the SalA/SalB contents in four “concentrated extract” products (labeled potency: 5 × 20 ×) were 0.126–0.951 μg/mg [17]. SalA concentrations in our samples were obviously higher than the reported values.

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### Fig. 3
Stability of SalA/SalB in the extracts with and without the GCP treatment. The extracts were stored at 4 °C without light shielding. Each data point represents mean ± S.D. (n = 3).
Smoking of 200–500 µg of SalA produces profound hallucinations lasting up to 1 h [19]. This potency is similar to the highly active synthetic hallucinogen LSD. Based on the SalA concentrations in our samples, it is estimated that smoking approximately 40–70 mg of the S. divinorum dried leaf products would be sufficient to cause hallucinogenic effects. On the other hand, the smoking dosage of the “concentrated extracts” products was dependent on their potencies. The range of the estimated dosage was from approximately 5 mg (sample #4, 2×) to 50 mg (sample #12, 25×). Considering the low estimated dosage, high-concentrated samples such as samples #10–#12 have a high risk of intoxication or accidents.

4. Conclusion

We have developed an improved analytical procedure for SalA and SalB in S. divinorum. This procedure was successfully applied to the determination of SalA and SalB in S. divinorum-related products. The present results indicate that all products contained levels of SalA high enough to cause hallucinogenic effects. In particular, some “concentrated extracts” products contained very highly concentrated SalA. These results will be very useful for increasing our understanding of drugs circulated in the Japanese illicit drug market.

References