Quantitative Determination of Incarvillateine in *Incarvillea sinensis* by Solid Phase Extraction and High Performance Liquid Chromatography

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Method for rapid quantitative analysis of incarvillateine in *Incarvillea sinensis* by high-performance liquid chromatography (HPLC) has been developed. The sample preparation involves solid phase extraction (SPE) with a mixed-mode reversed-phase and cation-exchange cartridge. The linear calibration range for incarvillateine was 0.002—0.5 mg/ml. The limit of detection was 0.35 μg/ml (S/N=3). Intra- and interday precisions were less than 0.36% (n=6) and 1.61% (n=18), respectively. The recovery of incarvillateine was 97.61—102.44% with the relative standard deviation (RSD) ranging from 0.63 to 1.93% (n=3). This method was proposed as a simple, rapid and accurate method for quantitative determination of incarvillateine content in various samples of *Incarvillea sinensis* collected from different areas of China.

Key words Bignoniaceae; *Incarvillea sinensis*; incarvillateine; solid phase extraction (SPE); HPLC

Incarvillea sinensis LAM, a well-known traditional Chinese crude drug, has been used to treat rheumatism and to relieve pain. We have examined the constituents of this crude drug in order to reveal the active principles, and obtained a number of novel monoterpene alkaloids and macrocyclic spermine alkaloids. Their chemical structures were identified by means of mass measurements, various NMR techniques and X-ray analyses.[1—8] In parallel with the chemical investigation, the antinociceptive effect was also been examined, and it was found that the main constituent of this crude drug, incarvillateine (Fig. 1), showed significant antinociceptive effect. When compared to the action of morphine, incarvillateine demonstrated more potent antinociceptive effect in the formalin test, and the mechanism of antinociception was believed to be different from that of morphine.[9]

No quantitative determination method has yet been established yet. We developed and validated a rapid and accurate HPLC method for the quantitative assessment of incarvillateine. This assay could be used to determine the incarvillateine contents in various samples of *Incarvillea sinensis*. Incarvillateine is not commercially available, and thus had to be isolated from the plant materials for reference purposes.

Experimental

Reagents and Materials Acetonitrile and methanol were of HPLC grade and purchased from Kanto Chemicals (Tokyo, Japan). Potassium dihydrogenphosphate was of analytical grade and bought from Wako Pure Chemicals (Osaka, Japan). Purified water was prepared by a Millipore Simpli Lab UV (Japan Millipore Ltd., Tokyo). Samples of *Incarvillea sinensis* were collected in Wuan and Handan, Hebei province, and in Qingdao and Zaozhuang, Shandong province of China in August from 2002 to 2004, and identified by the fifth author. A voucher specimen has been deposited at the Herbarium of Beijing University of Traditional Chinese Medicine and Pharmacy.

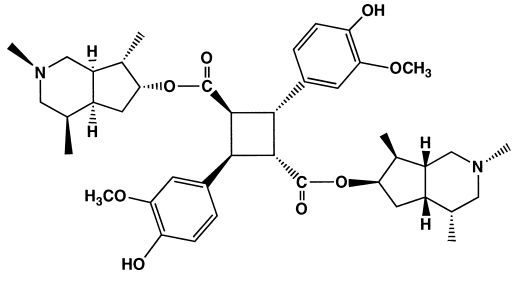

Fig. 1. Structure of Incarvillateine

Apparatus and Chromatographic Conditions The chromatographic separation was performed on a Hewlett-Packard (HP) Agilent 1100 series HPLC system (Agilent, Yokogawa Analytical Systems, Tokyo) with a diode array detector operating at 234 nm. The column used in this study was YMC-Pack Pro C18 RS (5 μm, 250×4.6 mm i.d.) (YMC Co. Ltd., Japan). The mobile phase was CH_3CN–40 mM KH_2PO_4 (22 : 78) and the flow rate was maintained at 1 ml/min. The column temperature was controlled at 35 °C. Data collection and manipulation were performed using HP Chem Station software for HPLC analyses.

Isolation of Incarvillateine from *I. sinensis* Aerial parts (5 kg) were extracted twice with 95% EtOH for 2 h and the combined extracts concentrated to a syrup at 60 °C. The residue was dissolved in 1% HCl, filtered, ammonia solution added to pH 10—11, and the alkaloids extracted into $CHCl_3$. After drying the solvent was removed to leave a residue weighing 24 g. The residue was applied to a column of silica gel which was then eluted with cyclohexane–EtOH–Et_2NH (50 : 1 : 1→5 : 1 : 1). Fractions were collected and the composition of each was monitored by TLC on silica gel G with benzene–acetone–methanol (7 : 2 : 1). Incarvillateine was obtained from eluates 524—544, and was repeatedly recrystallized from methanol. The product purity was confirmed by chromatographic methods (TLC and HPLC). The structure of incarvillateine was characterized by spectroscopic and X-ray analyses. mp 217.2—217.7 °C, $[\alpha]_D$ −10.8° (MeOH); EI-MS m/z: 718 $[M]^+$ (4), 360 (33), 359 (15), 183 (13), 182 (100), 166 (27), 58 (19); ^{1}H-NMR ($CDCl_3$) δ: 0.58 (1H, m, 6-Ha), 0.60 (3H, d, J=7.3 Hz, 8-Me), 0.71 (3H, d, J=6.7 Hz, 4-Me), 0.76 (3H, d, J=6.7 Hz, 4′-Me), 0.81 (3H, d, J=7.0 Hz, 8′-Me), 1.06 (1H, m, 6′-Ha), 1.42 (2H, m, 1, 1′-Ha), 1.58 (4H, m, 3, 3′-Ha, 6, 6′-Hb), 1.72 (2H, m, 8, 8′-H), 1.84 (2H, m, 9, 9′-H), 1.97 (2H, m, 4, 4′-H), 2.13 (2H, m, 5, 5′-H), 2.17 (6H, s, N, N′-Me), 2.45 (2H, m, 3, 3′-Hb), 2.55 (2H, m, 1, 1′-Hb), 3.81—3.89 (2H, m, β, β'-H), 3.88, 3.89 (each 3H, s, O, O′-Me), 4.30—4.38 (2H, m, α, α'-H), 4.89 (2H, m, 7, 7′-H), 6.77 (6H, m, 2″, 2‴, 5″, 5‴, 6″, 6‴-H); ^{13}C-NMR ($CDCl_3$) δ 14.4, 14.8 (8, 8′-Me), 16.8, 17.0 (4, 4′-Me), 29.1, 29.6 (6, 6′-C), 30.1 (4, 4′-C), 37.2 (5, 5′-C), 40.1 (8, 8′-C), 45.5, 45.7 (9, 9′-C), 45.9, 46.0 (N, N′-Me), 57.1, 57.3 (1, 1′-C), 57.4 (3, 3′-C), 76.2, 76.5 (7, 7′-C), 40.3, 41.7 (α, α'-C), 47.2, 47.8 (β, β'-C), 171.7, 171.9 (COO, COO′), 55.6, 55.7 (3″, 3‴-OMe), 110.8, 110.9 (2″, 2‴-C), 114.7 (5″, 5‴-C), 119.8, 120.3 (6″, 6‴-C), 130.2, 130.4 (1″, 1‴-C), 145.3, 145.5 (3″, 3‴-C), 146.8, 146.9 (4″, 4‴-C); X-ray analysis: triclinic system, space group $P1$; parameters are: a=6.585(3) Å, b=9.270(5) Å, c=17.244(7) Å, α=82.03(4)°, β=96.61(3)°, γ=109.05(4)°, V=982.7(8) Å^3, Dc=1.213 g/cm^3, Z=1; observed reflections were refined to R=0.08.

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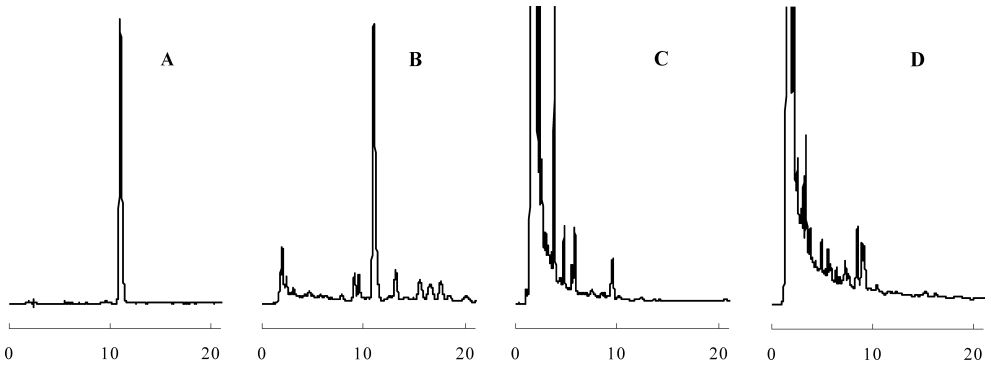

Fig. 2. HPLC Chromatograms of Incarvillateine

(A) Incarvillateine standard; (B) incarvillateine in the aerial parts of *Incarvillea sinensis* determined by the proposed method; (C) sample that flowed out from the cartridge for pretreatment; (D) washing solvent that passed through the cartridge after loading of sample ino it.

Preparation of Standard Solution A stock solution of incarvillateine was prepared in methanol at a concentration of 1 mg/ml. The solution was diluted with mobile phase to obtain a series of standard solutions with concentrations of 0.002, 0.01, 0.05, 0.1, 0.2 and 0.5 mg/ml. Linearity of the responses was determined for six concentrations with three injections for each level. The calibration curve was based on the concentration (mg/ml, *x*-axis) to peak area (*y*-axis).

Sample Clean-Up Sample clean-up was performed using a solid-phase extraction (SPE) system. The sample extracting solution was applied on a SPE cartridge packed with 60 mg of mixed-mode polymeric sorbent of 30 μm particle size with reversed-phase and cation-exchange functionalities (Oasis® MCX, Waters Co., MA, U.S.A.). Following the extraction of solution, the SPE column was washed with 2 ml methanol, 2 ml water and 2 ml of 0.5 M ammonia solution. Incarvillateine was eluted with 4 ml of MeOH–28% NH_3 (95 : 5). The eluate was collected, evaporated to dryness *in vacuo*, and the residue was dissolved in 2 ml of the mobile phase for analysis.

Results and Discussion

Incarvillateine is not commercially available, and it was first isolated from *Incarvillea sinensis* by us, and the structure was determined by spectroscopic and X-ray analyses.

Optimization of Extraction Conditions Powdered aerial parts (1 g) were extracted with different solvents, 50 ml of MeOH, 0.2% HCl or 0.2% HCl in 30% MeOH, respectively. MeOH extract (5 ml) was evaporated to dryness *in vacuo*, the residue redissolved in 5 ml of 0.2% HCl in 30% MeOH and submitted to SPE, while 0.2% HCl or 0.2% HCl in 30% MeOH extract (5 ml) was directly submitted to SPE. Furthermore, the same samples (1 g) were extracted with 50 ml of 0.2% HCl in 30% MeOH for different periods of 10, 20 or 30 min, respectively. The individual extracts obtained were submitted to SPE directly. For reasons of convenience and efficiency of extraction, 0.2% HCl in 30% MeOH was given preference, and a 96.4% extraction rate is achievable within 10 min.

Sample Clean-Up An efficient sample clean-up was achieved by solid phase extraction over a mixed-mode polymeric sorbent with reversed-phase and cation-exchange functionalities. The clean-up of samples using an Oasis® MCX cartridge consists of the following three steps: sample loading, washing and elution of incarvillateine from the cartridge. When the sample was loaded and passed through the cartridge, incarvillateine was trapped in the cartridge due to its basic properties. The cartridge was then washed with methanol, water and 0.5 M ammonia solution as described before. The remaining UV-absorbing phenolic constituents, organic acids and pigments were removed by this washing process, while incarvillateine was completely retained. After washing, incarvillateine was eluted with MeOH–28% NH_3 (95 : 5) as an elution solvent. Elution with 4 ml or more of the elution solvent resulted in a complete extraction of incarvillateine.

Typical chromatograms for incarvillateine extracted by the proposed method are shown in Fig. 2. Standard and extracted incarvillateine are shown as a single and sharp peak with the same retention time of about 11 min. When the sample was passed through the Oasis® MCX cartridge, no incarvillateine remained in the sample, nor was any in the washing solvent that flowed out of the cartridge (Fig. 2).

Method Development and Validation The proposed method for quantitative analysis of incarvillateine was validated in terms of linearity, intra- and interday precisions, accuracy and recovery.

Linearity was examined with the standard solutions prepared in the range of 0.002—0.5 mg/ml of incarvillateine. The linear relationship between the concentrations (mg/ml, *x*-axis) and peak area (*y*-axis) was expressed by the following equation: $y=7151.2x+2.4675$. The correlation coefficient was 1.0 and the calibration curve was a straight line. The limit of detection was 0.35 μg/ml (S/N=3) for incarvillateine.

Intra- and interday precisions were evaluated by replicate injection of a standard solution and a sample solution (Wuan, 2004). Six injections per day were conducted on days 1, 2 and 5 after sample preparation to determine reproducibility (after measurement, the solution was stored at 8 °C). The intraday precision of standard solution was found to be RSD 0.14% ($n=6$), and the interday precision was RSD 0.45% ($n=18$). Similarly, the peak areas of incarvillateine were measured and the intra- and interday precisions in sample solution were found to be RSD 0.36% ($n=6$) and 1.61% ($n=18$), respectively. This result shows that the standard and sample solutions were stable for at least 5 d when stored at 8 °C.

In order to examine the accuracy of the method as well as the recovery of extraction, 0.25, 0.50 and 0.75 mg incarvillateine were spiked into the samples of powdered aerial parts (1 g), and the samples were subjected to the extraction procedure as described before. As a result, the recoveries of extraction obtained were 97.61—102.44% with RSD ranging from 0.63 to 1.93% ($n=3$).

Comparison of Incarvillateine Content in Different

Table 1. Content of Incarvillateine in Various Samples of Aerial Parts Collected from Different Areas and in Different Parts of the Plant Determined by HPLC (Values are the Mean of 3 Samples)

Sample	Content of incarvillateine (mg/g)
Aerial parts	
Collected in Wuan, Heibei Province	
in 2002	1.6184±0.0182
in 2003	1.6348±0.0098
in 2004	1.5029±0.0003
Collected in Handan, Heibei Province	
in 2002	1.3588±0.0162
in 2003	1.4281±0.0083
in 2004	1.5676±0.0270
Collected in Qingdao, Shandong Province	
in 2002	1.4232±0.0038
in 2003	1.2829±0.0241
in 2004	1.4951±0.0054
Collected in Zaozhuang, Shandong Province	
in 2002	1.4721±0.0038
in 2003	1.4929±0.0069
in 2004	1.3457±0.0206
Different parts of the plant (Wuan, 2004)	
Leaves	4.7700±0.0139
Stems	0.1868±0.0021
Fruits	0.0994±0.0011

Samples The contents of incarvillateine in 15 samples were determined using the above developed HPLC method. The samples collected from different areas of China displayed variable contents of incarvillateine ranging from 1.2829 to 1.6348 mg/g (Table 1). The content of incarvillateine in leaves was about 25- and 48-fold higher than that in stems and fruits.

In conclusion, a rapid and robust HPLC assay for quantitative analysis of incarvillateine in *I. sinensis* has been developed and validated. Efficient removal of interfering substances (phenolic constituents, organic acids and pigments) was achieved by means of a SPE clean-up on a mixed-mode reversed-phase and cation-exchange cartridge. The clean-up of samples was very simple. The method is thus suitable for routine analysis. The content of incarvillateine in leaves was much higher than those in stems and fruits. Thus, the content of incarvillateine in this crude drug was affected significantly by the ratio of leaves in the plant materials.

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