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Short communication

A rapid and simple determination of protoberberine alkaloids in cortex phellodendri by ^{1}H NMR and its application for quality control of commercial traditional Chinese medicine prescriptions

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Abstract

Huangbai (cortex Phellodendri, the dried bark of *Phellodendron amurense* or *Phellodendron chinense*) is one of the important traditional Chinese medicines. Protoberberine alkaloids were reported to contribute to the biological activity of this species. A highly specific and sensitive method using ^{1}H NMR has been developed for the quantitative determination of protoberberine alkaloids in *Phellodendron* species and their commercial traditional Chinese medicine prescriptions. In the region of δ 8.6–8.9, the signals of H-13 of berberine (**1**) and palmatine (**2**), were well separated from other signals in methanol-d_4. The quantity of the compounds was calculated by the relative ratio of the integral values of the target peak of each compound to the known amount of internal standard anthracene. This method allows rapid and simple quantization of protoberberine alkaloids from *Phellodendron* species or the more complex commercial prescriptions in 5 min without any pre-purification steps. The recoveries of berberine and palmatine from *P. amurense* were in the range of 95–106%. Limit of detection (LOD) and limit of quantitation (LOQ) of them were 1.0 and 1.8 μg/mL, respectively. The advantages of the method were that no reference compounds are required for calibration curves, the quantification could be directly realized on a crude extract, the better selectivity for protoberberine alkaloids and a very significant time-gain could be achieved, in comparison to conventional HPLC methods, for instance.

Keywords: Protoberberine; Berberine; Palmatine; *Phellodendron*; ^{1}H NMR

1. Introduction

Phellodendron is a small genus of aromatic deciduous trees of East Asia often having thick corky bark. This bark has found application in Chinese traditional medicine for various diseases like meningitis, bacillary dysentery, pneumonia, tuberculosis and liver cirrhosis. Huangbai (cortex Phellodendri), the dry bark of *Phellodendron amurense* or *Phellodendron chinense* (family Rutaceae), was considered to be one of the 50 fundamental herbs in Chinese herbalism. It was commonly used in traditional Chinese medicine to remove damp heat, quench fire, counteract toxicity, relieve consumptive fever and also effective in curing dysentery, diarrhea and other syndromes [1]. *P. amurense* or *P. chinense* extracts and decoctions have demonstrated significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, helminthes and chlamydia [1]. Protoberberine alkaloids (Fig. 1) are considered to contribute for the biological activity of this species [2]. The pharmacological actions of protoberberine alkaloids include metabolic inhibition of certain organisms, inhibition of smooth muscle contraction, reduction of inflammation and stimulation of bile and bilirubin secretion. Currently, the predominant clinical uses of protoberberine alkaloids include bacterial diarrhoea, intestinal parasite infections and ocular trachoma infections [2,3]. Although these alkaloids were not consid-

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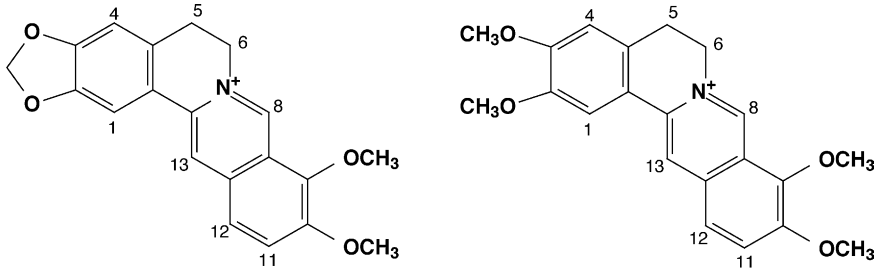

Fig. 1. The structure of berberine (**1**) and palmatine (**2**).

ered to be toxic at doses used in clinical situation, side effects can result from high dosages which include constipation, dyspnoea, lowered blood pressure, flu-like symptoms and cardiac damage. Berberine usage should be avoided in pregnancy, as it caused uterine contractions and miscarriage, and in jaundiced neonates because of its bilirubin displacement properties. Huangbai used in oriental medicine show quite variable quality, because of the two very similar *Phellodendron* species comprise the source of the Huangbai on the market. Moreover, the diverse geographical origins of the plants make the content of active alkaloids quite different from each other. It is necessary to determine the species in Huangbai used in commercial prescriptions and the contents of protoberberine alkaloids in plant material or prescriptions.

The quantitative methods for the determination protoberberine alkaloids in *Phelloderdon* species based on high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) have been described [4–6]. Controlling pH values of the mobile phase was needed to increase the resolution. Gas chromatography (GC) analysis required reduction of protoberberine alkaloids to the relative tetrahydroberberines [7]. Thin layer chromatography densitometry [8] and immunoquantitative analysis methods [9] were also reported for the analysis for protoberberine alkaloids. However, disadvantages, such as the low sensitivity, time-consuming and inextricable preparation steps and the need for extensive derivatization steps, remain problematic in the reported methods. For better controlling of these pharmaceutically important alkaloids in *Phellodendron* species and commercial prescriptions, a suitable method would be highly desirable.

Recently, high-resolution nuclear magnetic resonance (NMR) spectroscopy is developing into an important tool in quality control of phyto-preparations [10–14] and in clinical diagnosis and monitoring of treatment [15]. The advantages of ^{1}H NMR method are manifold, viz. it is rapid, noninvasive and does not require any sample pre-clean steps. In addition, no standard compounds are required for preparing the calibration curves and it detects all the components present in herbal preparations simultaneously in a single measurement. Therefore, we hypothesized that NMR spectroscopy may be superior to the conventional HPLC for the analysis of protoberberine alkaloids. In this paper, we described the quantitative analysis of protoberberine alkaloids from *Phellodendron* species and their commercial traditional Chinese medicine prescriptions (*shang zhong xia tong yong tong feng wan* [for treatment of rheumatic fever], *ban xia bai zhu tian ma tang* [for treatment of headache and gastroptosis], *yi qi cong ming tang* [for treatment of vertigo and amnesia] and *qing shu yi qi tang* [for treatment of heat-stroke and anorexia]) using ^{1}H NMR spectroscopy. This method allows rapid and simultaneous determination of two protoberberine analogues without any pre-cleaning steps.

2. Experimental

2.1. Chemicals

First grade methanol and anthracene were purchased from E. Merck (Germany). Methanol-d_4 (99.9%) was obtained from Aldrich (U.S.A.). The reference compounds (berberine and palmatine) were isolated from the roots of *P. amurense* in a prior study [16]. The purity of reference compounds was checked by NMR and HPLC methods (>99.7%).

2.2. Materials

The dried bark of *P. chinense* and *P. amurense* were collected from Bureau of Food and Drug Analysis, Department of Health, Executive Yuan, Taiwan, ROC, in August 2004 and verified by Prof. C.S. Kuoh. The commercial traditional Chinese medicine prescriptions (*shang zhong xia tong yong tong feng wan*, *ban xia bai zhu tian ma tang*, *yi qi cong ming tang* and *qing shu yi qi tang*) were purchased from Kaiser Pharmaceutical Company (Tainan, Taiwan), Chuang Song Zong Pharmaceutical Company (Kaohsiung, Taiwan), Sun Ten Pharmaceutical Company (Taipai, Taiwan) and Sheng Chang Pharmaceutical Company (Taipai, Taiwan).

2.3. Sample extraction

2.3.1. Method 1

A sample of 10 mg of powdered plant material (cortex of *P. chinense*) was extracted with 0.5 mL of MeOH using sonication at room temperature for 30 min (three

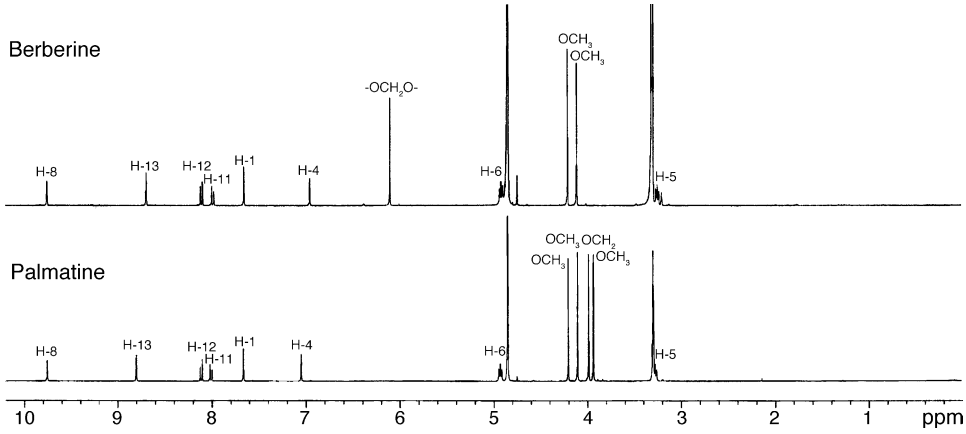

Fig. 2. The ^{1}H NMR spectra of berberine (**1**) and palmatine (**2**) in methanol-d_4.

times). The combined extracts were evaporated to dryness. The residue was dissolved in 0.5 mL MeOH-d_4 (contained 84.4 μg anthracene) and used for ^{1}H NMR measurement.

2.3.2. Method 2

A sample of 10 mg of powdered plant materials (cortex of *P. chinense* and *P. amurense*) or commercial prescriptions were exactly weighed into a NMR tube (0.3 mm i.d.) and added 0.5 mL of MeOH-d_4 (contained 84.4 μg anthracene). The sample was sonicated at room temperature for 30 min and used for ^{1}H NMR measurement.

2.4. NMR analysis

^{1}H NMR spectra were recorded in methanol-d_4 (99.9 %) using a Varian UNITY plus 400 MHz spectrometer. For each sample, 100 scans were recorded with the following parameters: 0.187 Hz/point; spectra width, 14400 Hz; pulse width, 4.0 μs; relaxation delay, 2 s. For quantitative analysis, peak area was used and the start and end points of the integration of each peak were selected manually.

2.5. Recovery

Pure berberine (**1**) and palmatine (**2**) were spiked into 10 mg of powered cortex of *P. amurense*. The recovery sample was prepared following method 2. A blank recovery sample was prepared and analyzed for the comparison. Limit of detection (LOD) was evaluated at a signal-to-noise ratio of 3. Limit of quantitation (LOQ) was evaluated at a signal-to-noise ratio of 6.

3. Results and discussion

3.1. NMR detection

Since the protoberberine alkaloids were high polar, methanol-d_4 was used as the NMR solvent to ensure that all the extract can be dissolved and avoid the inference of phenolic hydroxyl signals. The ^{1}H NMR spectra of **1** and **2** are well documented in methanol-d_4 (Fig. 2). The analysis of the NMR spectra of **1** and **2** revealed that the proton H-13 resonating in an empty region of spectra as a singlet (H-13 of **1**: δ 8.69; H-13 of **2**: δ 8.79) could be used for quantification.

A suitable internal standard should be preferably a stable compound with a signal in a non-crowded region of the ^{1}H NMR spectrum. For this purpose, anthracene, with a signal at δ 8.44 and the integral value maintaining constant within 48 h, has been chosen. In the case of ^{1}H NMR quantitative analysis, calibration curves were not needed for quantification of the compounds because integration of the peaks was always proportional to the amount of the compound and the same for all compounds in ^{1}H NMR.

Table 1
Comparison of the extraction methods

Extraction method	Amount of alkaloids in the cortex of *P. amurense* (μg/10 mg)	
	Berberine	Palmatine
Method 1		
First extract	126.60 ± 1.77	133.75 ± 2.14
Second extract	11.52 ± 0.10	15.02 ± 0.10
Third extract	–	–
Method 2	140.69 ± 1.55	153.91 ± 1.08

Data are means ± S.D. of three replicates.

Table 2
Recovery of the ^{1}H NMR method

Analyte	Amount added (μg)	Recovery (μg)	RE (%)
Berberine	20	20.56 ± 0.52	102.8
	100	96.22 ± 2.47	96.2
	500	491.13 ± 14.94	98.2
Palmatine	20	21.03 ± 0.61	105.2
	100	95.89 ± 2.88	95.9
	500	493.65 ± 12.43	98.7

All experiments were based on triplicate measurement.

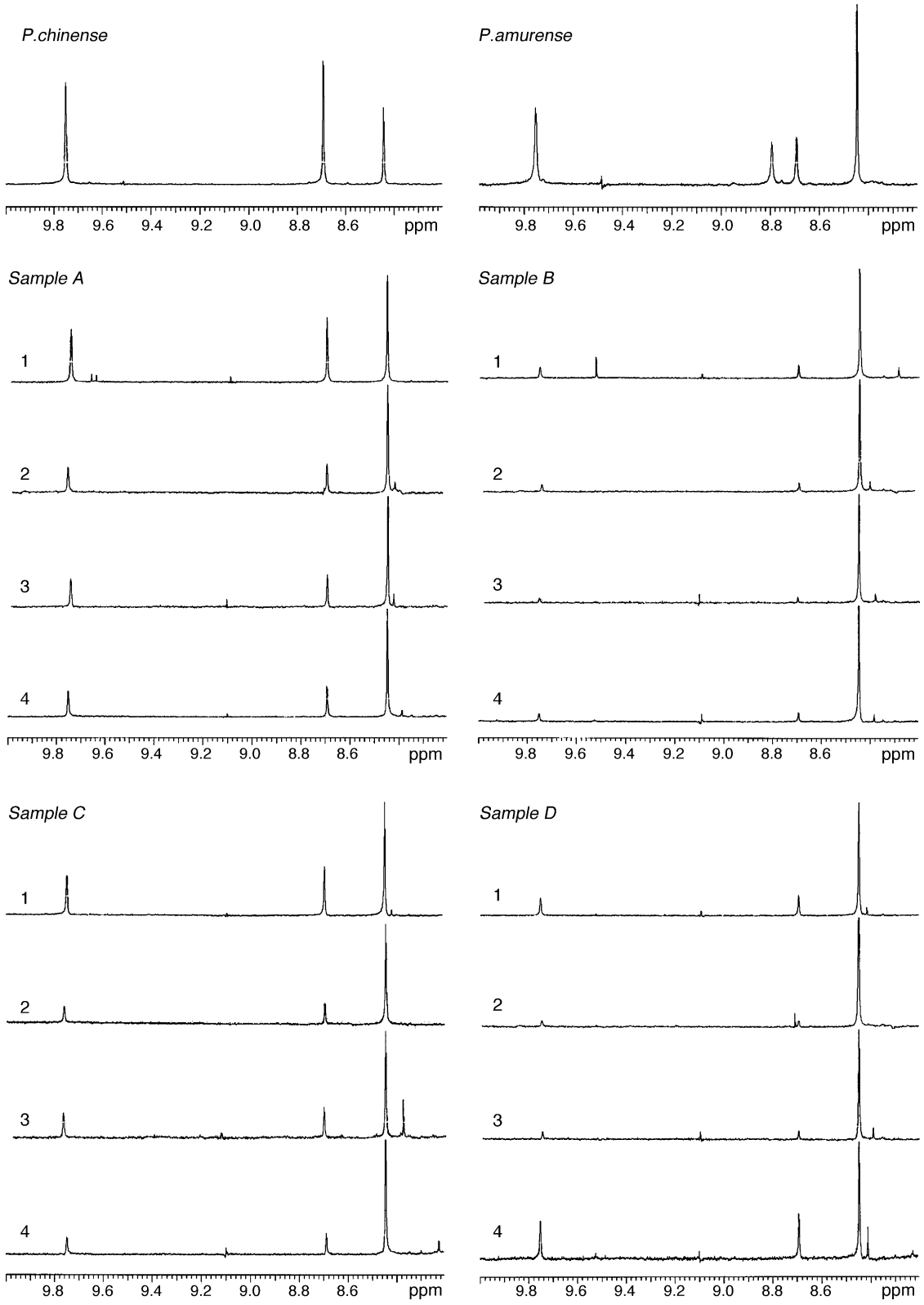

Fig. 3. ^{1}H NMR spectra of the cortex of *P. chinense*, *P. amurense* and Huangbai commercial prescriptions in the range of δ 10.0–8.2. Sample A: *shang zhong xia tong yong tong feng wan*; Sample B: *ban xia bai zhu tian ma tang*; Sample C: *yi qi cong ming tang*; Sample D: *qing shu yi qi tang*. **1**–**4**: company 1–4.

Table 3
The concentrations (mg/g) of berberine and palmatine in the cortex of *P. chinense, P. amurense* and Huangbai commercial prescriptions

Sample[a]	Berberine	Palmatine
P. chinense	68.25 ± 1.00	–
P. amurense	14.07 ± 0.27	15.39 ± 0.29

	Company 1	Company 2	Company 3	Company 4	
Sample A	21.61 ± 0.33	11.33 ± 0.16	10.18 ± 0.12	11.52 ± 0.15	–[b]
Sample B	4.45 ± 0.06	2.79 ± 0.05	1.64 ± 0.03	2.67 ± 0.05	–[b]
Sample C	15.36 ± 0.15	6.83 ± 0.11	9.98 ± 0.09	5.19 ± 0.04	–[b]
Sample D	6.94 ± 0.05	1.74 ± 0.03	2.73 ± 0.04	8.05 ± 0.14	–[b]

Sample A: *shang zhong xia tong yong tong feng wan*; Sample B: *ban xia bai zhu tian ma tang*; Sample C: *yi qi cong ming tang*; Sample D: *qing shu yi qi tang*.
[a] All experiments are based on triplicate measurement.
[b] In all samples obtained from four companies plamatine was not observed in the NMR spectra.

3.2. Comparison of extraction methods

In the current assay, two extraction methods were compared by analyzing replicate samples with the same lot number to find the most efficient and reliable one. As shown in Table 1, there was no significant difference in the extraction efficiency of these two target compounds between methods 1 and 2. However, the time using method 2 was shorter than method 1 and it was more simple and easy to prepare. Thus, method 2 was chosen for the sample analysis.

3.3. Recovery test

Three sets of recovery samples were analyzed by method 2 as described above. A blank recovery sample (without adding standards) was prepared and analyzed for comparison. The average recovery was observed between 96.2–102.8 and 95.9–105.2% for berberine (**1**) and palmatine (**2**), respectively (Table 2). Using this method, the LOD for berberine (**1**) and palmatine (**2**) was determined to be 0.8 μg/mL and the LOQ was found to be 1.8 μg/mL.

3.4. Sample analysis

Using the ^{1}H NMR method Hungbai materials including *P. amurense* and *P. chinense*, and 16 samples of four Huangbai commercial prescriptions from four different companies were analyzed for alkaloids, berberine (**1**) and palmatine (**2**). Quantification of **1** and **2** by ^{1}H NMR is possible by means of the integral of a well-separated specific proton signal of the compounds. For this purpose, the H-13 proton singlet of each alkaloid was selected as target peak as it was quite well separated from the others and could be integrated at this condition because it was in a region δ 8.6–8.9 where no interference from other signals occurred. The ^{1}H NMR spectra of Huangbai materials was shown in Fig. 3. In the ^{1}H NMR spectra, it was observed that *P. amurense* exhibited the H-13 signal of two alkaloids in the ratio of 1:1, but *P. chinense* displayed only H-13 signal of berberine. This observation can be used as identification manner to identify the species used in the commercial Huangbai preparation easily. In the present study, all the Huangbai prescriptions were identified to contain *P. chinense* as their source rather than *P. amurense*, since they displayed only H-13 signal of berberine (**1**).

The quantitation of protoberberine alkaloids, berberine and palmatine in the two *Phellodendron* species and various Haungbai samples determined by this ^{1}H NMR method were shown in Table 3. *P. chinense* was found to contain berberine as the major constituent. Besides, *P. amurense* contained berberine and palmatine in a ratio of about 1:1, but the total protoberberine alkaloids concentration was lower than *P. chinense*. The various commercial Huangbai prescriptions produced by different companies were showed a quite different content of berberine (**1**). All these preparation samples did not showed palmatine (**2**) content in their ^{1}H NMR spectra.

4. Conclusion

This NMR method is simple and rapid, specific, no reference compounds are needed, apart from the cheap internal standard and an overall profile of the preparation can be obtained directly. Using this method the contents of protoberberine alkaloids can be determined in a much shorter time than the conventional chromatographic or other analysis methods reported, and moreover without any derivatization. The described ^{1}H NMR method could be used as a rapid and simple method for the identification of *Phellodendron* species used in the Huangbai preparation as source and quantification of protoberberine alkaloids in plant materials or commercial prescriptions.

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