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DETERMINATION OF TETRANDRINE, FANGCHINOLINE, CYCLANOLINE AND OBLONGINE IN RADIX STEPHANIAE TETRANDRAE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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A reversed-phase high-performance liquid chromatographic method to simultaneously measure the four alkaloids [tetrandrine, fangchinoline, cyclanoline and oblongine] in Radix Stephaniae tetrandrae has been successfully developed. The method uses standards of the four compounds as external standards. These compounds were isolated in our laboratory using various chromatographic methods and are completely separated within 45 min using a reversed-phase column and linear gradient elution with dihydrogenphosphate buffer HPLC-grade acetonitrile mobile phase. The quantitative calibration curves are linear over a range of 12.5-1637 ì g/ml for all four compounds. The detection limits (S/N=3) for tetrandrine, fangchinoline, cyclanoline and oblongine are approximately 0.95, 0.95, 0.95 and 1.69ì g/ml, respectively.

Key words: HPLC, Radix Stephaniae Tetrandrae, Tetrandrine, Fangchinoline, Cyclanoline, Oblongine.

INTRODUCTION

The Chinese traditional medicine Radix Stephaniae tetrandrae, the root of *Stephania tetrandra* S. Moore (Menispermaceae), have been demonstrated to have anti-inflammatory, anti-allergic and hypotensive effects in experimental animals[1]. A large number of compounds have been isolated from this plant over the past fifty years in the search for the biologically active constituents[2-8]. Most of these compounds are alkaloids, which can be classified as bisbenzylisoquinoline, protoberberine, morphinane and phenanthrene types. The main constituents in

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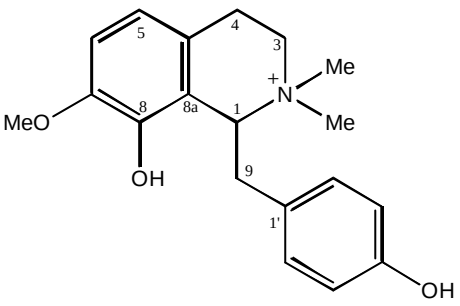
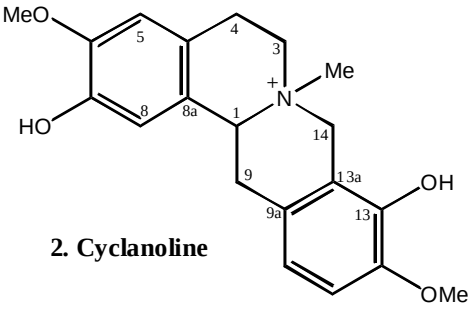
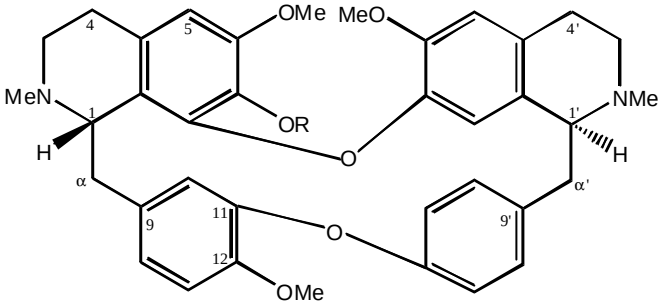


1. Oblongine



2. Cyclanoline



3. Fangchinoline, R = OH

4. Tetrandrine, R = OMe

Fig.1. Structures of the four alkaloids, oblongine (1), cyclanoline (2), fangchinoline (3) and tetrandrine (4) in Radix Stephaniae tetrandrae

Radix Stephaniae tetrandrae are tetrandrine and fangchinoline. Many pharmacological studies carried out on the purified compounds from the roots have suggested that the biological activity of the plant is mainly attributable to the alkaloids. Among the alkaloids, tetrandrine is best characterized as a Ca^{++}-entry blocker[9] although it also exhibits cardiovascular disorders modulating[10], anti-tumor[11] as well as anti-inflammatory effects[12]. Previously we found that the ethanolic extract of the roots could practically account for the cardioprotective and anti-inflammatory effects attributed to tetrandrine[12–13] suggesting that active principles in addition to tetrandrine may also be found in the roots. Therefore a standardized method for the analysis of the ethanolic extract of the roots is as important as the discovery of other active principles in the plant. We have isolated four alkaloids: tetrandrine, fangchinoline, cyclanoline and oblongine from the ethanolic extract of the roots and their structures were shown in Fig. 1. In this study a direct high-performance liquid chromatographic method for the simultaneous determination of these four alkaloids was developed.

EXPERIMENTAL

Reagents and materials

HPLC-grade acetonitrile was purchased from Tedia (Ohio, USA), sodium dihydrogenphosphate, phosphoric acid and silica gel from Merck (Germany). Sephadex LH-20 was purchased from Pharmacia Biotech (Uppsala, Sweden). Water was purified by a Milli Q system from Millipore (Milford, MA, USA). Radix Stephaniae tetrandrae was purchased from the Chinese herbal market in Taipei (Taiwan). The four standard alkaloids, tetrandrine, fangchinoline, cyclanoline and oblongine were isolated from the roots using chromatographic methods described below.

Isolation of the four standard alkaloids

Radix Stephaniae tetrandrae (610 g) was ground up and extracted with 95% EtOH three times at 80 °C (each 1000 ml, 8 h). The combined extract was concentrated by rotary evaporation under vacuum at 50 °C to dryness. 3% HCl (200ml) solution was added to the residue which was then extracted with $CHCl_3$ (200 ml × 3). The acid solution was adjusted to pH 9 with 25% NH_4OH and the resultant suspension was extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated to give tetrandrine and fangchinoline. The NH_4OH layer was then partitioned with n-BuOH. The n-BuOH layer was concentrated and the residue (5.1 g) was chromatographed on Sephadex LH-20 column with MeOH to give three fractions (I, II, III). Fraction II, which contained mostly cyclanoline, was recrystallized with MeOH to give cyclanoline as a grayish-white powder. The parent liquid of fractions II and III were combined and were subjected to column chromatography over silica gel eluting with $CHCl_3$-MeOH (9:1) to afford cyclanoline and oblongine. The four alkaloids were identified by comparing the IR, MS, ^1H- and ^{13}C- NMR spectral data with those contained in the literature.[4,14–15].

Preparation of sample solution

A 100 g pulverized Radix Stephaniae tetrandrae was extracted five times with EtOH (1500 ml, successively) by reflux at 80 °C, for 5 h each time. The extracts were combined and filtered and then evaporated in vacuum at ca. 50 °C to give 10.23g of residue. A 30mg portion of dried extract was dissolved in 1.5 ml of MeOH. The solution was filtered through a 0.45 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA) before use.

Apparatus and conditions

HPLC was performed on a Hitachi Model L-7100 Intelligent pump system equipped with a Hitachi Model L-7000 interface, a Hitachi Model L-7450A photodiode array detector and a Hitachi Model L-7200 auto-sampler. Detection wavelength was set at 280 nm. The separations were obtained with a reversed-phase column (Cosmosil

5C18-AR-II, 4.6 × 250 mm, Kyoto, Japan) eluted at a rate of 1 ml/min with a linear solvent gradient of A and B [A = KH_2PO_4-H_2O, 1000 ml: 5 g, pH adjusted with 8.5% H_3PO_4 to 2.91; B = H_2O-CH_3CN-KH_2PO_4, 400 ml : 600 ml : 5 g, pH adjusted with 8.5 % H_3PO_4 to 3.30] according to the following profile: 0-20 min, 72% A, 28% B; 20-55 min, 72-30% A, 28-70% B; 55-60 min, 30%-0% A, 70%-100% B; 60-80 min, 0% A, 100% B; 80-85 min, 0-72% A, 100-28% B; 85-100 min, 72% A, 28% B. The injected volume was 20 µl of the prepared solution.

Preparation of standard solution and calibration

To prepare a standard solution, an accurately weighed amount of the four standard alkaloids, tetrandrine, fangchinoline, cyclanoline and oblongine were dissolved in MeOH. Calibration curves were established based on five points covering a concentration range of 12.5-250 ìg/ml for tetrandrine, 12.5-250 ìg/ml for fangchinoline, 163.7-1637.5 ìg/ml for cyclanoline, 145-1450 ìg/ml for oblongine. The standard solution (20 ìl) were used for HPLC injections (n = 5) . Calibration graphs were plotted based on to linear regression analyses of the responses in peak areas in response to concentrations of standards injected.

Preparation of recovery studies

Three different concentrations each of the standard alkaloids; 708, 683 and 593 µg/ml for tetrandrine, 398, 373 and 360 ìg/ml for fangchinoline, 800, 636 and 571 µg/ml for cyclanoline, 307, 168 and 110 µg/ml for oblongine were added to each sample solution, respectively. All samples were filtered through a 0.45 µm syringe filter (Gelman) and injected for HPLC analysis and the concentrations of tetrandrine, fangchinoline, cyclanoline and oblongine calculated from their calibration curves.

RESULTS AND DISCUSSION

Development of the HPLC method

A variety of solvents were tested for their abilities to separate the four alkaloids present in the plant extract. Gradient systems of MeOH-H_2O or MeCN-H_2O in combination with (NH_4) H_2PO_4 buffer on a reversed column (Cosmosil 5C18-AR-II, 4.6 × 25 mm) did poorly in resolution. The addition of an ion-pair reagent (SDS) did not result in complete separation. Eventually, gradient systems of MeCN-H_2O in combination with KH_2PO_4 buffer at pH 2.91-3.00 (profile described in section 2-4) were used to achieve complete separation on the same reversed-phase column (Fig. 2). The sensitivity of the analysis was examined by comparing UV spectra of the four alkaloids as shown in Fig. 3. Because solutions of tetrandrine, fangchinoline, cyclanoline and oblongine exhibited shown a characteristic absorption at 280nm, we selected 280 nm as the appropriate wavelength for detection and quantification. The HPLC system should be completely washed for 20 min after each run to maintain column reproducibility.

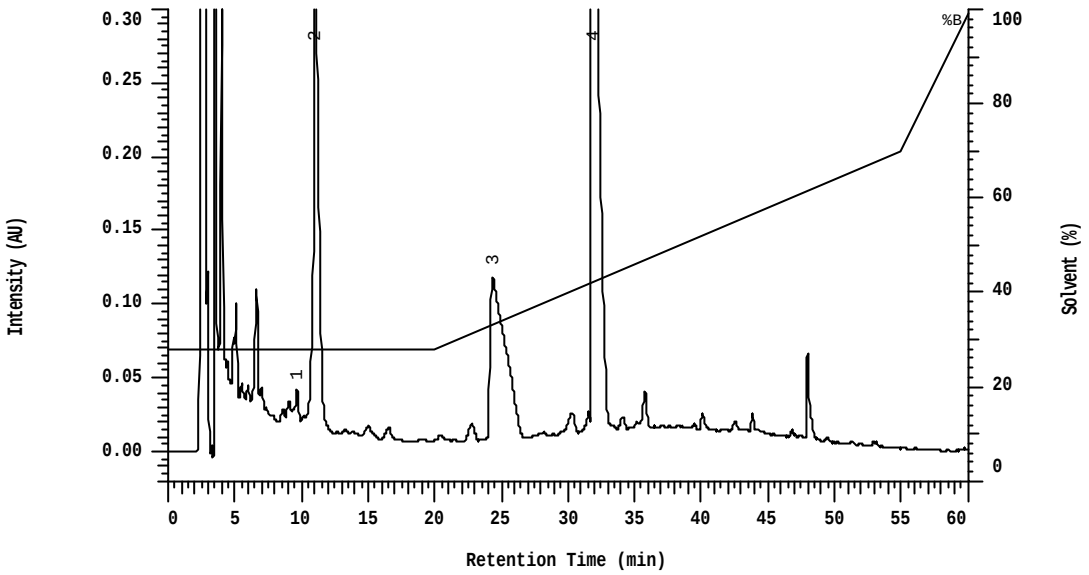


Fig. 2. Chromatogram of ethanolic extract of Radix Stephaniae tetrandrae . Peak identities: oblongine (1), cyclanoline (2), fangchinoline (3) and tetrandrine (4)

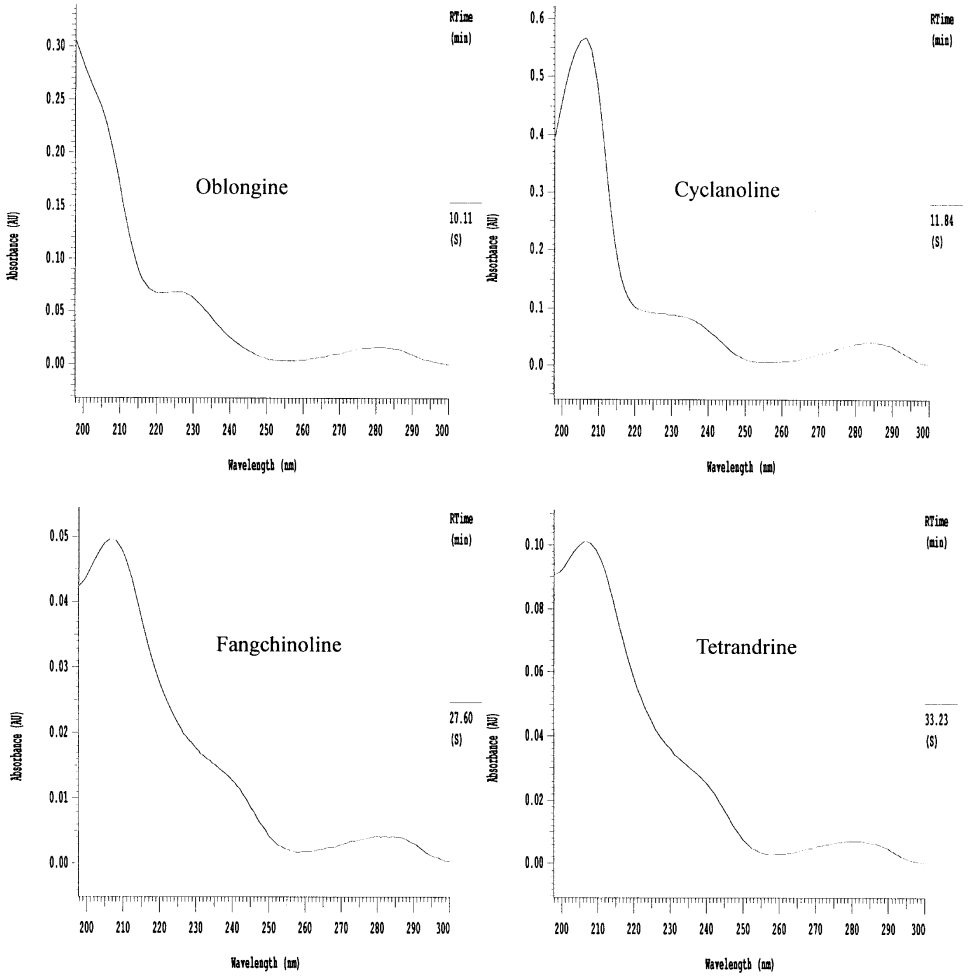


Fig. 3. On-line UV spectra of the four alkaloids in Radix Stephaniae tetrandrae

Table 1. Intra-day and inter-day assay variations of four standard alkaloids

Alkaloid substance	Amount measured (μg/mL)		Mean ± S.D. (R.S.D.,%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Oblongine	719.2 717.1 708.3 720.6 717.1	697.9 717.2 742.2 735.1 733.4	716.5 ± 4.8 (0.67)	728.4 ± 21.1 (2.90)
Cyclanoline	781.1 782.3 782.4 786.5 783.1	765.1 787.3 798.3 800.6 800.9	783.1 ± 2.0 (0.26)	793.9 ± 19.5 (2.46)
Fangchinoline	104.8 106.2 108.4 109.0 104.7	104.6 104.0 104.7 104.1 102.3	106.6 ± 2.0 (1.88)	103.9 ± 1.0 (0.96)
Tetrandrine	94.7 96.1 96.7 95.4 96.4	101.1 95.6 100.9 97.9 97.1	95.9 ± 0.8 (0.83)	98.5 ± 2.4 (2.44)

Calibration graphs

Calibration graphs were constructed in the range of 12.5-250 μg/ml for tetrandrine, 12.5-250 μg/ml for fangchinoline, 163.75-1637.5 μg/ml for cyclanoline and 145-1450 μg/ml for oblongine. The regression equations of these curves and their correlation coefficients were calculated as follows: tetrandrine, y = 0.00020x-0.5304 (r = 0.9999); fangchinoline, y = 0.00021x-0.5334 (r = 0.9998); cyclanoline, y = 0.00028x-1.555 (r = 0.9999); oblongine, y = 0.00076x-1.475 (r = 0.9999).

System suitability test

To assess the precision of these methods, we injected standard solutions of tetrandrine, fangchinoline, cyclanoline and oblongine, respectively, five times on the same day for intra-day and analyses over 6-day period for inter-day variations. The relative standard deviations (R.S.D.s) of intra-day and inter-day analyses were less than 8.0 and 8.0%, respectively. The precision as well as accuracy of this assay was satisfactory (Table 1). The results of the recoveries of tetrandrine, fangchinoline, cyclanoline and oblongine ranged from 63.6 to 89.4% (Table 2).

Table 2. Recoveries of four alkaloids in Radix Stephaniae tetrandrae

Alkaloid ubstance	Amount measured (ì g/mL)	Amount added (ì g/mL)	Recovery (%)	Mean±S.D. (%)	R.S.D (%)
Oblongine	213.8	307	69.66		
	104.3	168	62.08	63.57 ± 4.06	6.39
	64.9	110	58.95		
Cyclanoline	709.1	800	88.64		
	547.3	636	86.05	86.11 ± 1.68	1.96
	477.6	571	83.64		
Fangchinoline	337.8	398	84.87		
	316.5	373	84.85	83.81 ± 1.41	1.68
	294.1	360	81.69		
Tetrandrine	636.2	708	89.86		
	614.5	683	89.97	89.42 ± 0.66	0.74
	671.0	593	88.42		

Table 3. Reproducibility of separation of four alkaloids in Radix Stephaniae tetrandrae

Alkaloid substance	R.S.D. (%) (n = 3)	
	Retention time	Amount measured
Oblongine	1.00	2.42
Cyclanoline	0.92	0.94
Fangchinoline	1.52	2.40
Tetrandrine	0.32	0.33

Detection limit and precision

The detection limit was measured as the lowest concentration corresponding to a signal-to-noise ratio of 3:1. According to this rule, the values for tetrandrine, fangchinoline, cyclanoline and oblongine were approximately 0.95, 0.95, 0.95 and 1.69 ì g/ml, respectively. The reproducibility (R.S.D.) of three replications was 0.33-2.42%. The R.S.D. values of the retention time of each peak for three replicate injections were 0.32-1.52%. Data for individual compounds are shown in Table 3.

Determination of the four alkaloids in Radix Stephaniae tetrandrae

When the test solutions were analyzed by HPLC under the selected conditions, the contents of tetrandrine, fangchinoline, cyclanoline and oblongine in Radix Stephaniae tetrandrae were 82.31 ± 0.003, 43.50 ± 0.024, 59.09 ± 0.009 and 2.90 ± 0.024 mg/g, respectively. These results indicated that the HPLC method developed was suitable for the simultaneous determination of the tertiary and the quaternary alkaloids in Radix Stephaniae tetrandrae. Moreover, no sample pretreatment was needed for this method.

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REFERENCES

1. Jiangsu-xinyixueyuan, Zhongyao-dazidan (Encyclopedia of Chinese Materia Medica), Shanghai-renmin-chubanshe, Shanghai, pp 981-985, 1977.
2. Chen KK, Chen AL. The alkaloids of han-fang-chi. J Biol Chem 109:681-685, 1935.
3. Feng YX, Chen H. Pharmacognostic and chemical identification of Fang Ji (Stephaniae tetrandrae). Chin J Pharm anal 5:28-31, 1985.
4. Tomita M, Kozuka M, Lu ST. Studies on the alkaloids of Menispermaceous plants. CCXXIX. Alkaloids of *Stephania tetrandra*. Yakugaku Zasshi 87:316-318, 1967.
5. Hu TM, Zhao SX The structure of oxofangchirine and stephenanthrine isolated from *Stephania tetrandra* S. Moore. Acta Pharm Sin 21:29-34, 1986.
6. Ogino T, Sato T, Sasaki H, Chin M, Mitsuhashi H. Four new bisbenzylisoquonoline alkaloids from the root of *Stephania tetrandra* (fen-fang-ji). Heterocycles 27:1149-1154, 1988.
7. Ogino T, Yamaguchi T, Sato T, Sasaki H, Sugama K, Okada M, Maruno M. Studies on inhibitory activity against acetycholinesterase of new bisbenzylisoquinoline alkaloid and its related compounds. Heterocycles 45:2253-2260, 1997.
8. Ogino T, Katsuhara T, Sato T, Sasaki H. Okada M, Maruno M. New alkaloids from the root of *Stephania tetrandra* (fen-fang-ji). Heterocycles 48:311-317, 1998.
9. Felix JP, King VF, Shevell JL, Garcia ML, Kaczorowski GJ, Bick IR, Slaughter RS. Bis(benzylisoquinoline) analogs of tetrandrine block L-type calcium channels: evidence for interaction at the diltiazem-binding site. Biochemistry 31:11793-11800, 1992.
10. Huang YT, Hong CY. Tetrandrine. Cardiovasc Drug Rev 16:1-15, 1998.
11. DeConti RC, Muggia F, Cummings FJ, Calabresi P, Creasey WA. Clinical and pharmacological studies with d-tetrandrine. Pro Am Asso Cancer Res Am Soc Clin Oncol 16:96, 1975.
12. Shen YC, Chen CF, Wang SY, Sung YJ. Impediment to calcium influx and reactive oxygen production accounts for the inhibition of neutrophil Mac-1 up-regulation and adhesion by tetrandrine. Mol Pharmacol 55:186-193,1999.
13. Yu XC, Wu S, Wang GY, Shan J, Wong TM, Chen CF and Pang KT. Cardiac effects of the extract and active components of radix Stephaniae tetrandrae, II. Myocardial infarct, arrhythmia, coronary arterial flow and heart rate in the isolated perfused rat heart. Life Sci 68:2863-2872, 2001.

14. Lin LZ, Shieh LH, Angerhofer CK, Pezzuto JM, Cordell GA, Xue L, Johnson ME, Ruangrungsi N. Cytotoxic and antimalarial bisbenzylisoquinoline alkaloids from Cyclea barbata. J Nat Prod 56:22-29, 1993.
15. Moriyasu M, Ichimaru M, Nishiyama Y, Kato A. Isolation of alkaloids from plant materials by the combination of ion-pair extraction and preparative ion-pair HPLC using sodium perchlorate. I. Magnoliae Cortex. Natural Medicines 48:282-286, 1994.

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漢防己藥材中指標成分 Tetrandrine, Fangchinoline, Cyclanoline, Oblongine 之高效液相層析定量法之探討

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應用高效液相層析儀同時分析漢防己藥材中指標成分 tetrandrine, fangchinoline, cyclanoline **及** oblongine **之含量，採用** C_{18} **逆向層析管柱，移動相為乙腈-磷酸二氫鹽緩衝溶液，利用線性梯度沖提，檢測波長為** 280 nm**；以上述四種成分作為外插標準；在** 45 **分鐘內四種成分可完全分離，其定量線性回歸方程式及相關係數之濃度在** 12.5 1637 ì g/ml **範圍間，檢測極限依** S/N=3 **規例計算，四種標準品** tetrandrine, fangchinoline, cyclanoline **及** oblongine **分別為** 0.95, 0.95, 0.95 **及** 1.69 ì g/ml。

關鍵詞：高效液相層析法，漢防己， Tetrandrine, Fangchinoline, Cyclanoline, Oblongine。

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