Liquid chromatographic separation of the alkaloids in coptis–evodia herb couple

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Abstract

A total of seven quaternary and ten tertiary alkaloids in the coptis–evodia herb couple were separated on a Cosmosil 5C$_{18}$-MS column (5 μm, 250×4.6 mm I.D.) using a mixture of methanol and sodium dodecyl sulfate–NaOAc–HOAc buffer or NaOAc–HOAc buffer as eluent, wherein the latter was found to be better for the analysis of coptis–evodia crude extract. Experiments showed that retention times of the quaternary alkaloids were mainly governed by the composition of buffer used and those of the tertiary alkaloids were chiefly determined by the content of organic modifier in the mobile phase. Linearity of around two orders in the magnitude of concentration was generally obtained and limits of detection for these compounds were in the range of 0.28–6.77 ng. The relative standard deviations of retention times were less than 1% ($n=6$). Contents of the coptis–evodia herb couple or Chinese herbal preparation containing this herb couple could easily be determined by the proposed method.

Keywords: Coptidis rhizoma; Evodiae fructus; Pharmaceutical analysis; Alkaloids

1. Introduction

Herbs used together in couples are the basic composition units of Chinese herbal formulas and have special clinical significance in traditional Chinese medicine. The herb couples are much simpler than complicated formulas in composition but retain the basic therapeutic features [1]. Coptis (*Coptidis rhizoma*) possesses the effects of dispelling heat, drying dampness, purging fever and removing toxins, and evodia (*Evodiae fructus*) has the actions of warming the middle torso, dispelling cold, causing

vitality to descend and controlling pain [2]. The combined use of the two herbs will enable the healing of hypochondric and costal pain, stomach ache, acid regurgitation, nausea and gastric upset [1]. Hence the two herbs are the basic components of a number of formulas. Thus, the development of a simple and expedient analytical method for simultaneously assaying the two herbs is necessary.

The pharmacologically active constituents of coptis are a number of protoberberine alkaloids [3–7], and those of evodia are indolequinoline and quinolone alkaloids [8–12], as shown in Fig. 1. The two herb drugs have been analyzed well by two distinctly different ion-pair reversed-phase high-performance liquid chromatographic (HPLC) methods
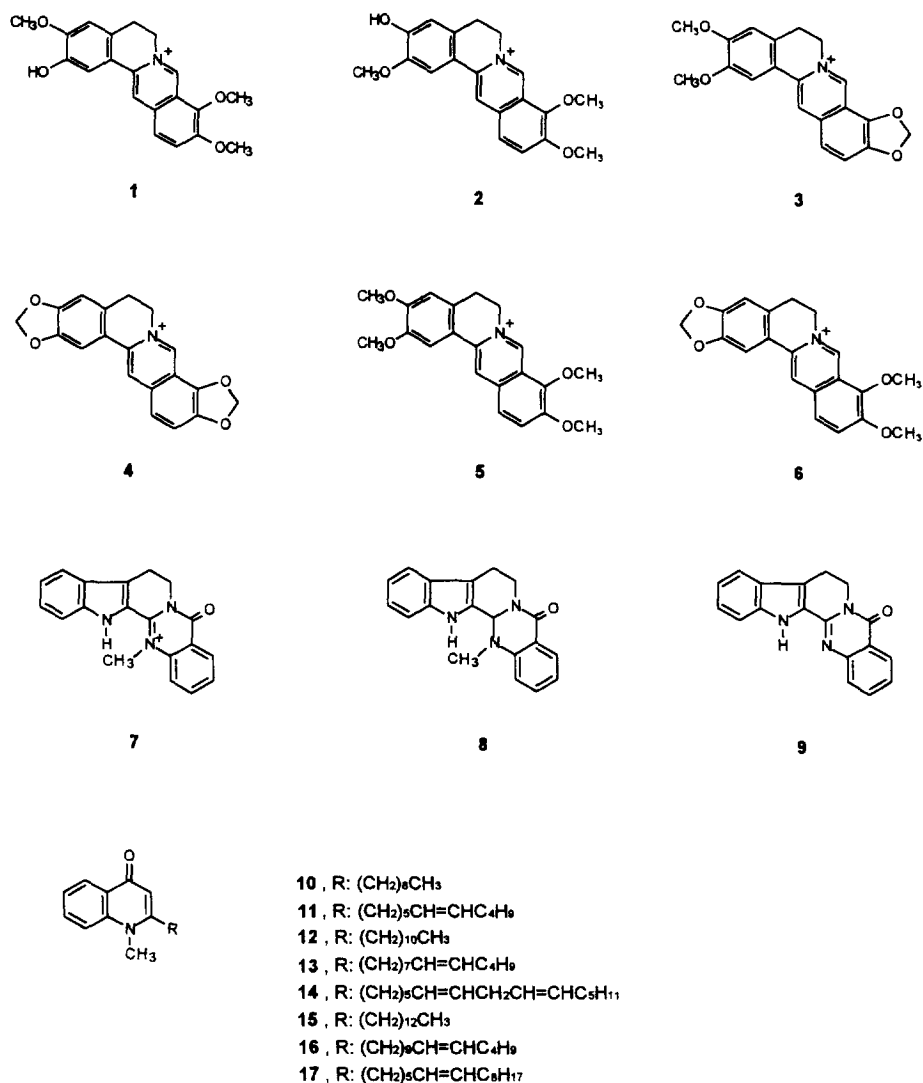
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Fig. 1. Structures of the 17 alkaloids: **1**, columbamine; **2**, jatrorrhizine; **3**, epiberberine; **4**, coptisine; **5**, palmatine; **6**, berberine; **7**, dehydroevodiamine; **8**, evodiamine; **9**, rutaecarpine; **10**, 1-methyl-2-nonyl-4(1H)-quinolone; **11**, 1-methyl-2-[(Z)-6-undecenyl]-4(1H)-quinolone; **12**, 1-methyl-2-undecyl-4(1H)-quinolone; **13**, evocarpine; **14**, 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)-quinolone; **15**, dihydroevocarpine; **16**, 1-methyl-2-[(Z)-10-pentadecenyl]-4(1H)-quinolone; **17**, 1-methyl-2-[(Z)-6-pentadecenyl]-4(1H)-quinolone.

[13,14]. However, none of them is practicable for analyzing the coptis–evodia herb couple. Here, we report two simple and rapid methods for separating the 17 alkaloids from a crude methanol–water extract of the herb couple. The suitabilities of the two methods were compared and discussed.

2. Experimental

2.1. Reagents and materials

The 11 evodia alkaloids (**7–17**) were isolated from *Evodiae fructus* [8–12] and epiberberine,

columbamine and jatrorrhizine from *Coptidis rhizoma* [3–7]. The structures of these compounds were elucidated on the basis of spectra data. Berberine chloride, palmatine chloride, β-naphthol and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA), coptisine chloride from Nacalai Tesque (Kyoto, Japan), sodium acetate and acetic acid from Merck (Darmastadt, Germany). Acetonitrile and methanol were of LC grade (Fisons, Loughborough, UK). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffer and sample solutions. *Coptidis rhizoma* and *Evodiae fructus* were purchased from the Chinese herbal market in Taipei, Taiwan. Purity checking and peak identification of all indexing standards and test samples was done with a photodiode array detector.

2.2. Preparing the extracts of herb couple and Chinese herbal preparation

A 0.4-g sample of the pulverized herb couple (containing 0.1 g *Coptidis rhizoma* and 0.3 g *Evodiae fructus*) was extracted with 70% methanol (10 ml) by refluxing for 15 min, then centrifuging at 1500 g (Universal, Hettich Zentrifugen) for 5 min. Extraction was repeated three times. The extracts were combined and added with a 1-ml internal standard solution (1.5010 g of β-naphthol in 500 ml of methanol), then diluted to 50 ml with 70% methanol. The methanol solution was filtered through a No. 1 filter paper and then a 0.45-μm filter. A 10-μl volume of the filtrate was injected into the HPLC system.

A 2.0-g sample of the constituent crude drugs of Pien-tung-wan (including 1.0 g *Coptidis rhizoma* and 1.0 g *Evodiae fructus*) was added to a 20-fold mass of water (40 ml) and boiled mildly for 40 min until the volume was halved. After filtration while hot, the filtrate was diluted with water to a volume of 50 ml. Placing 15 ml of this solution and 1 ml of internal standard solution into a 50-ml volumetric flask, the mixture was diluted with methanol to give a 70% methanol solution. The methanol solution was filtered through a No. 1 filter paper and then a 0.45-μm filter, and 10 μl of the filtrate was injected into the HPLC system.

2.3. Apparatus and conditions

The analysis was carried out on a HPLC system which consisted of two LC-6AD pumps, an SCL-6B system controller, a Rheodyne 7125 injector and an SPD-M6A photodiode array detector ($\lambda=250$ nm), all purchased from Shimadzu (Kyoto, Japan).

The separations were obtained by linear gradient, using eluents A and B. In this paper, there were two eluent systems, solvent system 1 (A1, B1) and solvent system 2 (A2, B2), to be discussed later: A1 = buffer–CH_3CN (60: 40) where the buffer was a solution consisting of 15 mM SDS, 50 mM NaOAc and 2.0% HOAc (pH 4.65); B1 = water–MeOH–HOAc (10:90:0.5, v/v). A2 = buffer–CH_3CN (80:20) where the buffer was a solution consisting of 50 mM NaOAc and 0.25% HOAc (pH 5.09); B2 = water–MeOH (10:90, v/v). Gradient programs used for the

Table 1
Gradient elution systems used for HPLC separations

Stage	Time (min)	Flow-rate (ml/min)	Gradient mode	Elution solvent (%)	
				A1	B1
System 1					
Equilibration	20	0.85	Step	100	0
Start	10	0.85	Linear	60	40
	15	0.85	Step	60	40
	5	0.85	Linear	25	75
	5	0.85	Linear	15	85
	15	0.85	Linear	0	100
Hold	5	0.85	Step	0	100
Regeneration	5	0.85	Linear	100	0
System 2					
				A2	B2
Equilibration	10	0.85	Step	100	0
Start	20	0.85	Linear	85	15
	5	0.85	Linear	55	45
	5	0.85	Linear	40	60
	5	0.85	Linear	15	85
	15	0.85	Linear	0	100
Hold	10	0.85	Step	0	100
Regeneration	5	0.85	Linear	100	0

A1: buffer–CH_3CN (60:40); buffer = 15 mM SDS, 50 mM NaOAc and 2.0% HOAc (pH 4.65). B1: water–MeOH–HOAc (10:90:0.5, v/v). A2: buffer–CH_3CN (80:20); buffer = 50 mM NaOAc and 0.25% HOAc (pH 5.09). B2: water–MeOH (10:90, v/v).

two solvent systems are shown in Table 1 and flow-rate was kept constant at 0.85 ml/min.

The column used was Cosmosil $5C_{18}$-MS, 5 μm, 250×4.6 mm I.D. (Nacalai Tesque). A precolumn of NovaPak silica (Millipore, Milford, MA, USA) was used not only to protect the column but also to increase the resolutions of some constituents in the coptis–evodia herb couple.

3. Results and discussion

3.1. Analytical conditions

During the study of *Evodiae fructus*, we examined a series of reversed-phase columns and found that Cosmosil $5C_{18}$-MS could give the best separation for the alkaloids [14]. Therefore, this column was chosen for the herb couple analysis.

3.1.1. Separation by solvent system 1

The quaternary alkaloids in *Coptidis rhizoma* have been determined successfully by an ion-pair reversed-phase HPLC (mixture of acetonitrile and a buffer solution consisting of 0.87 M HOAc, 0.12 M NaOAc, 11.56 mM SDS and 16.12 mM Et_2NH in the ratio of 2:3) in our laboratory [13]. However, this isocratic solvent system not only failed to separate the herb-couple constituents but also greatly reduced the column's life-span owing to the high HOAc and NaOAc concentrations used. Modifying the method mentioned above by reducing the acetate concentration in pump A and applying an aqueous methanol in pump B, a gradient solvent system (solvent system 1) for separating all the coptis–evodia alkaloids was developed.

First, four buffer systems containing 50 mM NaOAc and 2% HOAc at different SDS concentrations, 5, 10, 15 and 20 mM, were prepared in order to study the effect of SDS on separability, and thus the capacity factors (k') for the alkaloids at different SDS concentrations were obtained. The SDS dependence of the k' values of the quaternary alkaloids (1–7) was similar and as the SDS concentration increased, the capacity factor became larger. However, those of compounds 8–17 were not

influenced. From the results, with SDS concentration at 15 mM, all peaks were well separated.

The effect of NaOAc concentration on the separability with the five buffer solutions was examined (containing 15 mM SDS and 2% HOAc at five different NaOAc concentrations, 0, 30, 40, 50 and 60 mM). The capacity factors of all the quaternary alkaloids (1–7) became smaller as the NaOAc concentration increased. When NaOAc was not added or was present at concentrations below 10 mM, 2 and 3 overlapped completely or partially. At 30–60 mM, all peaks separated well but theoretical plate numbers varied dramatically. Compounds 1–3 increased (from 5354–18 265 to 25 147–33 300) and 5–7 decreased (from 112 718–158 131 to 8083–115 935) when NaOAc concentration increased. Taking into account the contents of 1–3 which were much lower than those of 5–7, the conditions which could give 1–3 a higher theoretical plate number should be selected. At 50 mM NaOAc, all peaks had the best resolution, meanwhile, 9 could be separated completely from an unidentified peak of the herb couple.

As we fixed the buffer solution at 15 mM SDS and 50 mM NaOAc as discussed above and varied the HOAc concentrations ranging from 1.0 to 2.5% (pH 4.95–4.52). The results showed that there were no apparent changes in capacity factors but there was some improvement in resolution, especially that between 5 and 6, when HOAc concentration increased. The R_s value for 5/6 was 0.85 at 1.0% and 1.27 at 2.0%. Therefore, 2.0% HOAc was chosen.

From the above results, the best resolution was achieved with the use of a buffer solution containing 15 mM SDS, 50 mM NaOAc and 2.0% HOAc and acetonitrile (3:2) in pump A. For pump B, acetonitrile and methanol were selected as organic modifier to establish a gradient system. When only acetonitrile was used, a good separation for the alkaloids was obtained except for 1 and 2, which were overlapped completely. Adding some methanol to the acetonitrile, however, a better resolution between 1 and 2 could be obtained. The R_s values for 1/2 pair at 40, 60, 80 and 100% methanol were 0.53, 0.89, 1.00 and 1.34, respectively. When 100% CH_3OH was used, a best resolution for 1/2 was obtained and the overlap of 9 and 12 with some unidentified peaks was

avoided. Finally, well-mixed methanol–water (9:1) was used to prevent the ghost peaks and a small amount of HOAc (0.5%, v/v) was added to eliminate the baseline drift.

A precolumn is mainly used to protect the column, and the packing material used therein normally complements that used in the analytical column. Our experiments revealed that precolumn of μBondapak C_{18} not only gave poor resolution for **1–3** and **5/6** (R_s: **1/2**, 1.16; **2/3**, 0.84; **5/6**, 0.83) but also provided lower theoretical plate numbers for **1–4** (N: **1**, $2.1 \cdot 10^4$; **2**, $1.8 \cdot 10^4$; **3**, $1.9 \cdot 10^4$; **4**, $1.0 \cdot 10^4$); an improvement was achieved by using silica (R_s: **1/2**, 1.34; **2/3**, 1.05; **5/6**, 1.27. N: **1**, $2.5 \cdot 10^4$; **2**, $2.1 \cdot 10^4$; **3**, $2.3 \cdot 10^4$; **4**, $2.1 \cdot 10^4$). Therefore, a precolumn of the NovaPak silica type was chosen in this study. Moreover, the HPLC system should be washed for a constant interval of time after each run in order to maintain the column's reproducibility.

A chromatogram showing the separation of methanol–water extract of coptis–evodia herb couple, using the proposed method, is shown in Fig. 2.

3.1.2. Separation by solvent system 2

Although solvent system 1 could be used to separate the coptis–evodia alkaloids well, some troubles existed, such as the variable retention times coming from different washing intervals applied, and the unstable analytical conditions owing to the
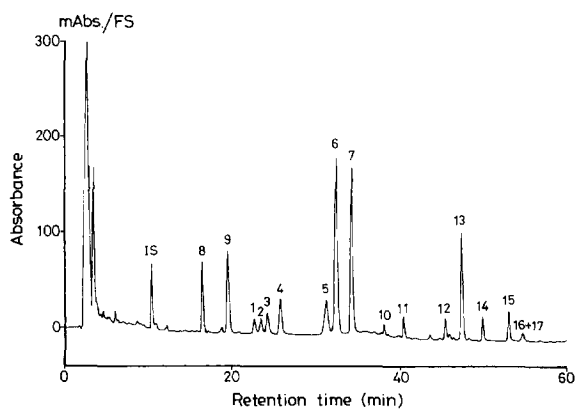

Fig. 2. HPLC chromatogram for the extract of a coptis–evodia herb couple separated by solvent system 1. Column: Cosmosil 5C₁₈-MS (250×4.6 mm I.D., particle size 5 μm). Detection: UV at 250 nm. I.S.=β-naphthol, other symbols are as in Fig. 1.

occurrence of gas bubbles in the system resulted from the use of SDS. Hence, the development of an alternative analytical method was necessary.

The alkaloids in *Evodiae fructus* have been determined by a linear gradient mobile phase with a simple NaOAc–HOAc buffer in pump A (mixture of acetonitrile and 30 mM NaOAc–1.25% HOAc buffer in the ratio of 1:4) [14]. Based on this method, a modified condition was developed for the determination of the 17 alkaloids. First, we prepared six eluent solutions containing 80% of 1.25% HOAc solution at different NaOAc concentrations ranging from 10 to 60 mM NaOAc (pH 3.91–4.39) and 20% of acetonitrile. Using these eluents, the capacity factors of the column for each alkaloid were obtained. Data in experiments shows that the k' values of **1–7** increased and **8–17** kept constant as NaOAc concentrations increased. At concentrations higher than 30 mM, all alkaloids could be separated, but 50 mM gave the best resolution for **1–3** (R_s: **1/3**, 1.14; **2/3**, 0.79) and the highest theoretical plate numbers for **6** ($1.03 \cdot 10^5$) and **7** ($2.12 \cdot 10^4$).

Five solvent systems containing 80% of 50 mM NaOAc buffer at 0, 0.25, 0.50, 1.25 and 3.00% HOAc (pH 7.66–4.01) and 20% of acetonitrile were used in order to study the effect of acetic acid concentration on the separability. As HOAc was absent, there was no dehydroevodiamine peak. The addition of HOAc to the buffer not only improved the resolution of **1/3** and **2/3** pairs greatly but also enlarged the theoretical plate numbers of **6** and **7** dramatically. From the results, at 0.25% HOAc, the resolution between **2** and **3** was best (R_s=0.96) and the theoretical plate number for **6** was highest (N= $1.10 \cdot 10^5$).

The composition of mobile phase in pump B was found to be the same as those in solvent system 1 except HOAc, which was not necessary in the present case. In addition, a NovaPak silica precolumn also gave better resolution and sharper peaks than that from C_{18} type. For example, the resolutions of **1/3**, **2/3**, and **4/7** were 1.33, 1.32 and 1.90 when silica was used, and were 0.97, 0.85 and 0.80 as C_{18} was applied.

A chromatogram showing the separation of methanol–water extract of the coptis–evodia herb couple, using solvent system 2, is shown in Fig. 3.
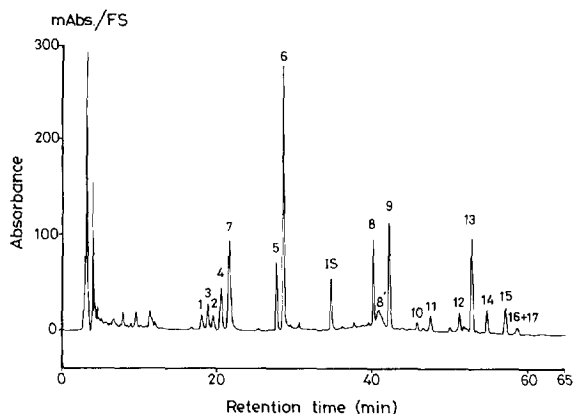
Fig. 3. HPLC chromatogram for the extract of a coptis–evodia herb couple separated by solvent system 2. The symbols as in Fig. 2.

3.1.3. Comparison between solvent systems 1 and 2

The alkaloids in the coptis–evodia herb couple can be grouped into two categories: tertiary alkaloids (8–17) and quaternary alkaloids (1–7). From the results shown in Figs. 2 and 3, it was found that the retention times of tertiary alkaloids were determined chiefly by the content of organic modifier in the mobile phase. As the modifier content raised to about 60% of the mobile phase (10 min in solvent system 1 and 30 min in solvent system 2), 8 and 9 would move out; around 80% (35 min in solvent system 1 and 40 min in solvent system 2), 10–12 could be eluted out; and at 90% (48 min in solvent system 1 and 50 min in solvent system 2), peaks of 13–17 appeared. However, peaks of quaternary alkaloids were determined mainly by the kind of buffer used. It is known that SDS not only could link much more tightly with positive nitrogen atoms of alkaloids to form a stable ion-pair than that of NaOAc did [15], but also would interact strongly with the stationary phase of C_{18} column [16]. As a result, the quaternary alkaloids had longer retention times in solvent system 1 than in solvent system 2, and the capacity factor of 1–7 increased in solvent system 2 but decreased in solvent system 1 when NaOAc concentration increased.

In our preliminary study, we found that the time for equilibration (run-to-run interval) might affect the reproducibility of the retention times. Therefore,

three sets of equilibration times (10, 20 and 30 min) were used to study the effect of the washing period on the reproducibility. The results indicated that the retention times of alkaloids varied with different equilibration times selected, especially those of 1–7 whose RSDs of retention time were in the range of 1.53–5.61% for solvent system 1 but less than 0.94% for solvent system 2. The shift in retention time resulted chiefly from the presence of SDS. This phenomenon not only affected the accuracy of analytical data but also caused the overlap of alkaloids with some unidentified minor peaks. In addition, the resolution of alkaloids in Fig. 3 (solvent system 2) was found to be much better than that in Fig. 2 (solvent system 1) except for 7 and 8. Compound 7 was a broader peak in solvent-system 2 and compound 8 was interfered with by the gradient drift (8′) in Fig. 3. Overall, solvent system 2 could offer a sharper peak, a higher resolution and a more stable retention time, therefore, this condition was chosen for the assay of the coptis–evodia herb couple.

3.2. Method validation

3.2.1. Precision

The reproducibility (relative standard deviation) of the proposed method (solvent system 2) in terms of the peak-area ratio in six replicate injections, was 0.02–1.79% (intra-day) and 0.13–2.01% (inter-day); the variation of retention time of each peak in the range of 0.01–0.09% (intra-day), and 0.01–0.94% (inter-day), $n = 6$. The detection limits ($S/N = 3$) for the alkaloids were 0.28–6.77 ng.

3.2.2. Linearity

Calibration graphs for the 17 alkaloids were obtained over a range of two orders of magnitude of concentrations. Results of the regression analyses and the correlation coefficients (r) are shown in Table 2. The data showed good linear relationships between peak-area ratios (y) and concentration (x, $\mu g/ml$).

3.2.3. Accuracy

Suitable amounts (0.26–11.55 mg, about 50% of the contents of the herb couple) of the 17 alkaloids were added to a sample of a herb couple of known

Table 2
Data of linear ranges, correlation coefficients (r) and recovery studies of the alkaloids

Compound	Linear range (μg/ml)	Slope (×10⁻³)	Intercept	r	Recovery (%)
1	0.29– 22.00	35.23	0.1445	0.9999	98.05
2	0.27– 20.00	35.23	0.1445	0.9999	97.89
3	0.30– 22.50	40.43	0.0172	0.9991	99.62
4	0.48– 36.00	42.45	0.0181	0.9991	101.58
5	1.00– 75.00	33.83	0.1388	0.9999	100.79
6	3.80–285.00	35.44	0.1454	0.9999	97.62
7	8.80–596.20	7.75	0.0041	0.9999	97.03
8	3.90–294.00	25.39	0.0098	1.0000	100.00
9	3.30–226.00	34.33	0.6177	0.9992	101.21
10	0.30– 19.70	30.57	0.0017	1.0000	97.30
11	0.90– 59.00	20.06	0.0053	1.0000	97.18
12	0.70– 50.20	28.80	0.0079	1.0000	97.29
13	3.60–243.50	29.45	0.0447	0.9999	98.30
14	1.10– 84.00	15.54	0.0025	0.9999	98.43
15	0.90– 57.90	28.95	0.0007	0.9999	98.60
16+17	0.40– 26.20	15.81	0.0002	0.9999	98.60

alkaloid content and the mixture was analyzed using the proposed procedure. The recoveries of the alkaloids were 97.2–101.2% ($n=3$), as shown in Table 2.

3.3. Determination of alkaloids in the herb couple and Chinese herbal preparation

When the test solution was analyzed by HPLC under the selected condition, the chromatogram shown in Fig. 3 was obtained. The peaks were identified by comparing the retention times and UV spectra with those obtained from authentic samples, and by spiking the mixture with a single constituent in subsequent run. By substituting the peak-area ratios of the individual peaks for y in the equations listed in Table 2, the contents of the individual alkaloids in the coptis–evodia herb couple and Chinese herbal preparation such as Pein-tung-wan were calculated. The results are given in Table 3. Data in Table 3 showed that there were much lower contents in the water-decoction extract than in the methanol–water extract, especially for those alkaloids (8–17) that were water insoluble or practically insoluble.

The HPLC method proposed for the separation and determination of tertiary and quaternary alkaloids may be conveniently used for quality assurance of commercial Chinese herbal preparations containing the coptis–evodia herb couple and also for quality control in pharmaceutical factories.

Acknowledgments

Table 3
Contents (mg/g crude drug) of the alkaloids in the herb couple and the Chinese herbal preparation (mean±SD; $n=3$)

Compound	Herb couple	Pien-tung-wan
1	1.78±0.04	1.50±0.00
2	1.76±0.06	1.51±0.00
3	2.00±0.05	1.85±0.00
4	3.53±0.06	2.87±0.02
5	5.40±0.08	4.64±0.07
6	18.05±0.20	13.66±0.11
7	53.63±0.25	16.35±0.18
8	7.99±0.04	0.59±0.04
9	4.98±0.03	0.92±0.01
10	0.59±0.01	0.04±0.01
11	1.89±0.00	0.10±0.01
12	1.60±0.06	0.10±0.00
13	8.61±0.03	0.43±0.04
14	3.98±0.03	0.21±0.00
15	2.67±0.01	0.14±0.01
16+17	1.44±0.02	–

– =The content was below detection limit.

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