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Short communication

Determination of the alkaloids in Evodiae Fructus by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of four indolequinazoline alkaloids (carboxyevodiamine, dehydroevodiamine, evodiamine and rutaecarpine) and eight quinolone alkaloids (1-methyl-2-nonyl-4(1H)-quinolone, 1-methyl-2-[(Z)-6-undecenyl]-4(1H)-quinolone, 1-methyl-2-undecyl-4(1H)-quinolone, evocarpine, 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)-quinolone, dihydroevocarpine, 1-methyl-2-[(Z)-10-pentadecenyl]-4(1H)-quinolone and 1-methyl-2-[(Z)-6-pentadecenyl]-4(1H)-quinolone) in Evodiae Fructus was developed. The method was carried out by using a Capcell C_{18} SG-120 column with a gradient solvent system of acetate buffer–acetonitrile–methanol and a UV detector (250 nm), and the contents of the alkaloids in a non-pretreated Evodiae Fructus extract could easily be determined within 40 min. Validation of this method is described.

Keywords: Evodiae Fructus; Alkaloids

1. Introduction

Evodiae Fructus, the unripe fruit of *Evodia rutaecarpa* (Juss.) Benth. or *E. rutaecarpa* (Juss.) Benth. var. *officinalis* (Dode) Huang, is a Chinese herbal drug which contains alkaloids, essential oils, carboxylic acids and limonoids as its main components. According to pharmacological tests, the alkaloids were found to have antifungal, analgesic, cardiotonic and body-temperature maintaining effects [1]. This study takes account of four indolequinazoline alkaloids, carboxyevodiamine (**1**), dehydroevodiamine (**2**), evodiamine (**3**), rutaecarpine (**4**) and eight quinolone alkaloids, 1-methyl-2-nonyl-quinolone (**5**), 1-methyl-2-[(Z)-6-undecenyl]-4(1H)-quinolone (**6**), 1-methyl-2-undecyl-4(1H)-quinolone (**7**), evocarpine (**8**), 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)-quinolone (**9**), dihydroevocarpine (**10**), 1-methyl-2-[(Z)-10-pentadecenyl]-4(1H)-quinolone (**11**) and 1-methyl-2-[(Z)-6-pentadecenyl]-4(1H)-quinolone (**12**), as shown in Fig. 1.

Kano et al. developed a high-performance liquid chromatographic method (HPLC) for simultaneous determination of three indolequinazoline alkaloids, hydroxyevodiamine (**2′**), **3**, and **4**, and four quinolone alkaloids, **6**, **7**, **8**, and **10**, in 130 min [2]. However, this method not only needs a quite long analysis time but also limits to just seven components of a pretreated test solution. We describe here the development of a direct and rapid method for determining twelve alkaloids in a crude Evodiae

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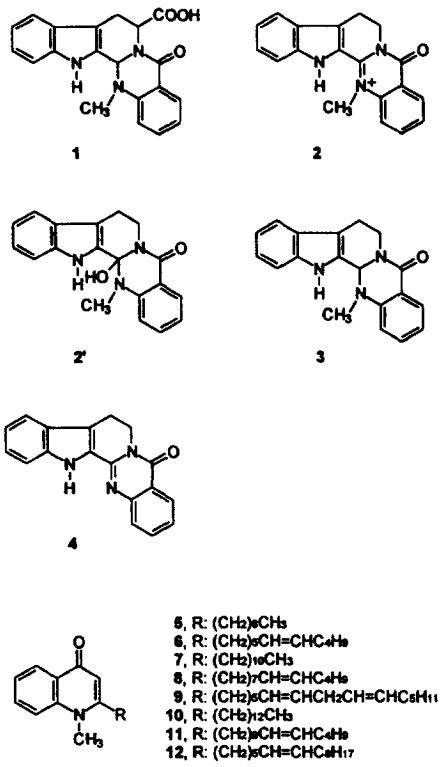

Fig. 1. Structures of the 12 evodia alkaloids. **1**, carboxyevodiamine; **2**, dehydroevodiamine; **3**, evodiamine; **4**, rutaecarpine; **5**, 1-methyl-2-nonyl-4(1H)-quinolone; **6**, 1-methyl-2-[(Z)-6-undecenyl]-4(1H)-quinolone; **7**, 1-methyl-2-undecyl-4(1H)-quinolone; **8**, evocarpine; **9**, 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)-quinolone; **10**, dihydroevocarpine; **11**, 1-methyl-2-[(Z)-10-pentadecenyl]-4(1H)-quinolone; **12**, 1-methyl-2-[(Z)-6-pentadecenyl]-4(1H)-quinolone.

Fructus extract within 40 min. The effects of mobile phase and column selectivity were also investigated.

2. Experimental

2.1. Reagents and materials

The alkaloids (**1–12**) were isolated from Evodiae Fructus [3–7], and were confirmed by comparing their IR, PMR, CMR and MS with those in the references. Sodium acetate and acetic acid were purchased from Merck (Darmstadt, Germany). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Acetonitrile and methanol were of LC grade (Mallinckrodt, Paris, KY, USA). Evodiae Fructus was purchased from the Chinese herbal market in Taipei (Taiwan). Purity checking and peak identification of all indexing standards and test samples were done with a photodiode array detector.

2.2. Preparation of Evodiae Fructus extract

A 0.3-g sample of pulverized Evodiae Fructus was extracted by refluxing with 70% methanol (7 ml) for 15 min, then centrifuged at 1500 *g* (Universal, Hettich Zentrifugen) for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After adding 1 ml of internal standard solution (0.5022 g cinnamic acid in 500 ml methanol), the Evodiae Fructus extract was diluted to 50 ml with 70% methanol; 10 μl of this solution were injected onto the HPLC system.

2.3. Apparatus and conditions

The HPLC system consisted of two LC-10AD pumps, a CBM-10A communication bus module, a Rheodyne 7125 injector and an SPD-M10A photodiode array detector (λ=250 nm), all purchased from Shimadzu (Kyoto, Japan).

The separations were obtained by linear gradient elution, using the eluents A and B [A: buffer–CH_3CN (80:20), buffer was a solution consisting of 30 m*M* NaOAc and 1.25% HOAc; B: H_2O–CH_3CN–CH_3OH–HOAc (10:45:45:0.25, v/v)] according to the following profile: 0–15 min, 100–30% A, 0–70% B; 15–22 min, 30% A, 70% B; 22–25 min, 30–0% A, 70–100% B; 25–40 min, 100% B. The flow-rate was kept constant at 1.0 ml/min.

The columns used are listed in Table 1. Precolumn of μBondapak C_{18} (Millipore, Milford, MA, USA) was used to protect the column.

Table 1
Columns used

No.	Column	Producer	Pore size (A)	Surface area (m^2/g)	Carbon content (%)	Bonding type[a]
I	Cosmosil $5C_{18}$	Nacalai tesque (Kyoto, Japan)	110	330	20	Mono
II	Cosmosil $5C_{18}$-MS	Nacalai tesque	120	300	16	Mono
III	Cosmosil $5C_{18}$-AR	Nacalai tesque	120	300	16	Polymer
IV	Inertsil ODS-80A	G.L. Science (Tokyo, Japan)	80	450	17.5	Mono
V	Capcell C_{18} SG-120	Shiseido (Tokyo, Japan)	100	320	15	Polymer-coated
VI	Purosphere RP-18	Merck (Darmstadt, German)	80	500	18.5	Mono

[a] Insert structures

3. Results and discussion

3.1. Analytical conditions

Kano et al. [2] reported a HPLC method for the simultaneous determination of seven evodia alkaloids by using an Inertsil ODS column with a gradient solvent system (H_2O–CH_3CN–HOAc–THF) in 130 min. However, efforts to try to shorten the analysis time by modifying their conditions were found to be invalid.

In our laboratory, the quaternary alkaloids in Coptidis Rhizoma have been well separated with either ion-pair reversed-phase HPLC or capillary zone electrophoresis by using acetate solution as buffer [8,9]. Similar conditions were found to be able to separate six evodia alkaloids in a number of processed Evodiae Fructus samples as well [10]. In the preliminary study, we found that the addition of acetate to the mobile phase and the selection of a suitable column are the two main determinant factors in achieving a good resolution for the separation of 12 evodia alkaloids in a crude methanol–water extract.

3.1.1. Mobile phase

Among the columns available in our laboratory, we found that Capcell C_{18} SG-120 (Shiseido) gave the best separation in our preliminary study. This column was therefore chosen as a basis of our research in searching for optimum eluent composition. First, we prepared nine HOAc solutions of different concentrations: 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00 and 2.50%. Using these eluents, the capacity factors (k') of the column for each alkaloid were obtained. The results indicated that all the evodia alkaloids except dehydroevodiamine (**2**) could be well separated no matter which eluent was used. By using a mobile phase without HOAc, no signal appeared for compound **2**. Actually, it spread over a range of 10 min (retention time from 33 to 43 min) and could be detected as a very low and broad peak in this condition if the detection wavelength was set at 370 nm. With the addition of acetic acid to the mobile phase, a much higher and narrower peak for **2** was achieved. Increasing the HOAc concentration from 0.25 to 2.50% not only narrows the peak-width of compound **2** but also clearly changes its retention time. At a concentration of 1.25%, the most satisfactory resolution was obtained.

Although the separation of evodia alkaloids had been obviously improved after adding HOAc to the mobile phase, efforts to eliminate the peak-tailing of compound **2** were still necessary. Experimental results showed that the addition of NaOAc to a 1.25% HOAc solution could increase not only the peak-symmetry but also the H/W value (peak height/peak width). The H/W values were found to vary from 9.26 to 24.03 (0 m*M*, 9.26; 2.5 m*M*, 14.82; 5.0 m*M*, 15.12; 10.0 m*M*, 18.92; 15.0 m*M*, 18.59; 20.0 m*M*, 20.42; 25.0 m*M*, 20.92; 30.0 m*M*, 24.03; 40 m*M*, 23.47). From the results, a 1.25% HOAc solution consisting of 30 m*M* NaOAc gave the best resolution (condition A). Using a 30 m*M* NaOAc solution as mobile phase without HOAc, however, a good result was obtained only for quinolones and not for dehydroevodiamine, a quaternary alkaloid, which became broad and short in the chromatogram. The

main difference between these two eluent systems might be their pH values. It was found that the pH was 3.84 for the former and 8.04 for the latter.

In order to investigate the relationship between column selectivity and mobile phase in more detail, two other eluent systems for eluent A were prepared. One was a mixture of 1.25% HOAc and acetonitrile (condition B) and another was a water–acetonitrile solution (condition C). After a series of experiments, it was found that there were no obvious differences for any of the compounds except compound **2**. The capacity factor and peak width of **2**, however, varied quite a lot when different mobile and stationary phases were used and the results are listed in Fig. 2.

The results in Fig. 2 show that the peak of compound **2** was very broad no matter which column was used when condition C was applied as eluent. However, great variations occurred after adding acetic acid (condition B) or acetate–acetic acid buffer (condition A) to the mobile phase. The peak shape and time reproducibility of this quaternary alkaloid could be remarkedly improved only if an optimal concentration of acetate buffer was selected.

The composition of the organic solvent in the mobile phase was also studied. When only acetonitrile was used both in eluent A and eluent B, broader bandwidth, lower resolution and worse baseline were obtained for the quinolone compounds (**5–12**). When the methanol concentration in eluent B was increased, the quinolone peaks became sharper. After a series of experiments, the best resolution was achieved when an equal volume of acetonitrile and methanol was used. When more than 50% methanol was used in eluent B, a good result was obtained only for the pure authentic standards and not for the Evodiae Fructus extracts owing to the overlap of quinolones with some unidentified components. When the composition of eluent B was fixed at 50:50 as discussed above and the methanol concentration was varied from 0 to 100% in eluent A, it was found that resolution deteriorated and retention time became longer for the indolequinazoline alkaloids, especially compound **1**. Hence, acetonitrile was the only organic solvent used in eluent A.

3.1.2. Column selectivity

The mobile phase and stationary phase, which are the factors governing HPLC separation, have mutual influences on each other [11]. We therefore fixed the concentration of buffer at 30 m*M* NaOAc and 1.25% HOAc for eluent A as discussed above and compared the selectivities of six commercial C_{18} columns (all 25 cm in length and 5 μm in particle size), as shown in Table 1. Correlation between the kinds of columns and the capacity factors (k') of alkaloids is listed in Table 2.

The data in Table 2 showed that compounds **3–9** could be well separated no matter which column was used. However, column I gave broader peaks and

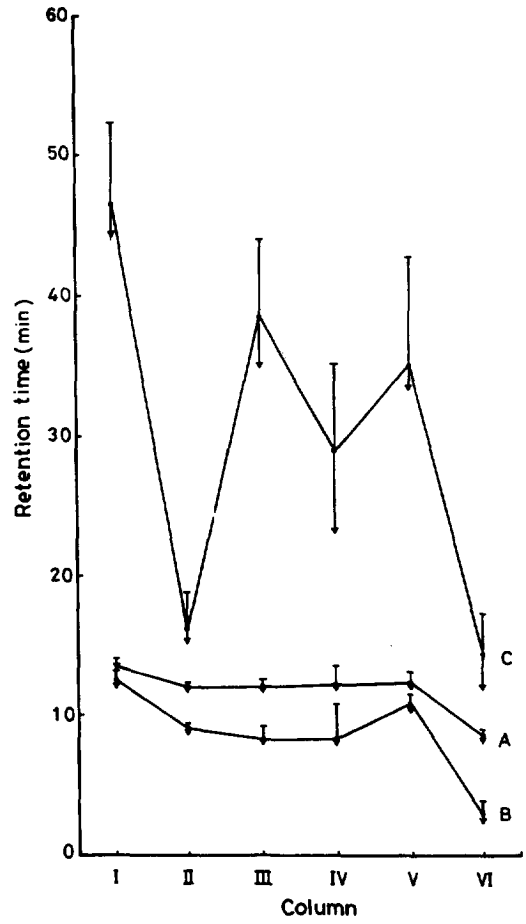

Fig. 2. The retention time of dehydroevodiamine obtained by using different columns and various mobile phases in eluent A. A: a buffer of 30 m*M* NaOAc and 1.25% HOAc/CH_3CN (80:20); B: 1.25% HOAc/CH_3CN (80:20); C: H_2O/CH_3CN (80:20). ⊤, peak-front; -●-, peak-apex; ↓, peak-tail. Column used: I, Cosmosil $5C_{18}$; II, Cosmosil $5C_{18}$-MS; III, Cosmosil $5C_{18}$-AR; IV, Inertsil ODS-80A; V, Capcell SG-120; VI, Purosphere RP-18.

Table 2
Correlation of column selectivity with capacity factor (k') of the alkaloids

Compound	I	II	III	IV	V	VI
1	2.4	2.0	2.0	2.5	2.1	3.5
2	3.7	3.0	3.1	3.8	3.1	2.7
3	7.0	6.2	6.3	7.8	6.0	8.0
4	7.9	6.8	7.1	8.8	6.5	8.7
5	10.1	9.2	9.5	11.1	9.0	12.0
6	10.4	9.5	9.8	11.5	9.3	12.3
7	11.7	10.3	10.6	12.6	9.9	13.3
8	12.1	10.6	10.8	13.0	10.2	13.5
9	12.6	11.0	11.1	13.5	10.4	13.8
10	14.5	12.0	12.2	15.0	11.2	15.3
11+12	14.9	12.3	12.4	15.5	11.4	15.3

lower resolutions, columns III and VI failed to separate **11** and **12** from **10**, columns IV and VI did not give satisfactory separation between **1** and some unidentified components. Both II and V gave good separations but with a shorter run time (3 min less) for column V. Therefore, column V was chosen for this analysis.

From the above results, the best resolution was obtained by using a Capcell C_{18} SG-120 column with a gradient solvent system of acetate buffer–acetonitrile–methanol. Fig. 3A presents a chromatogram of the 12 authentic alkaloids with the following retention times: **1**, 9.29 min; **2**, 12.35 min; internal standard (cinnamic acid), 14.42 min; **3**, 20.96 min; **4**, 22.47 min; **5**, 29.84 min; **6**, 30.78 min; **7**, 32.74 min; **8**, 33.36 min; **9**, 34.25 min; **10**, 36.56 min; **11+12**, 37.23 min. As the methanol–water extract of the Evodiae Fructus sample was injected directly and analyzed, the results were as good as those obtained with pure chemical samples without interference, as shown in Fig. 3B.

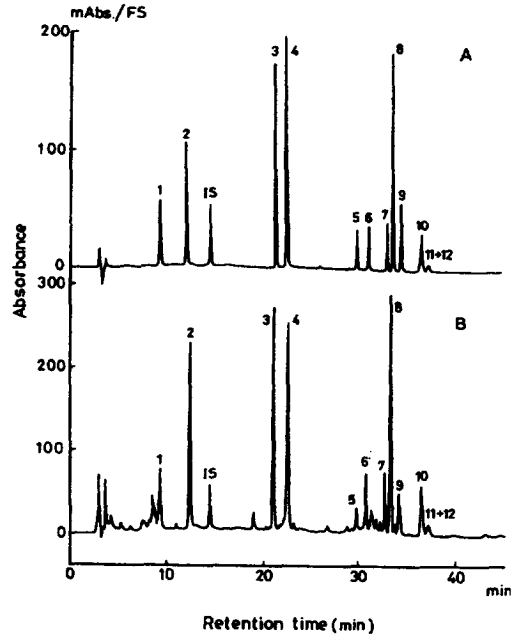

Fig. 3. HPLC chromatograms of (A) a mixture of the 12 evodia alkaloids, (B) the extract of an Evodiae Fructus sample.

3.2. Method validation

3.2.1. Identification

The 12 evodia alkaloids, **1–12**, in the samples were identified by comparing the retention times of authentic standards with those obtained from the sample chromatograms, and by spiking the mixture with a single alkaloid in a subsequent run. Hydroxyevodiamine had been previously reported as one of the major components in Evodiae Fructus [2]. However, there was no hydroxyevodiamine (**2′**) but dehydroevodiamine (**2**) in our samples after careful checking of the region where **2′** was supposed to be present. It is known that the UV spectrum of hydroxyevodiamine is almost identical with that of evodiamine, but is quite different from that of dehydroevodiamine which posses stronger absorbance and gives a rather bathochromic shift [4]. Fig. 4 was obtained by drawing the UV spectra of peaks **2**, **3** and **4** by photodiode array detector in all our test samples. From the results in Fig. 4, it can be seen that the structure of peak **2** should be similar to that of **4**. Hence, peak **2** was confirmed as dehydroevodiamine.

Compounds **11** and **12** are two structural isomers [6] and cannot be separated under our analytical conditions; hence, the total content of these two compounds was calculated.

3.2.2. Precision

The reproducibility (relative standard deviation) of the proposed method, on the basis of peak-area ratio for six replicated injections, was 0.30–1.61%, as shown in Table 3. The variation of retention time of

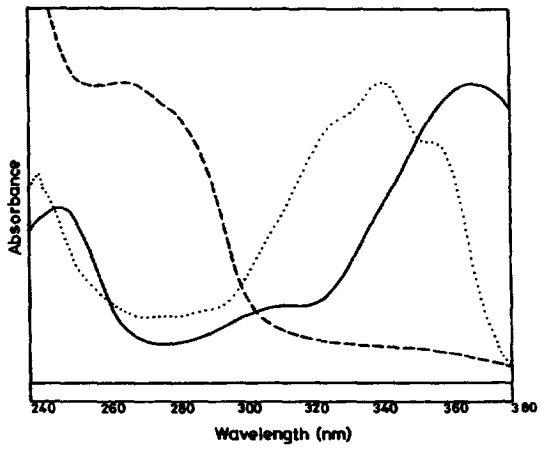

Fig. 4. Ultraviolet spectra of peaks **2**, **3** and **4**. (—) **2**, dehydroevodiamine (mAbs./FS.=506); (---) **3**, evodiamine (mAbs./FS.=290); (. . . .) **4**, rutaecarpine (mAbs./FS.=740).

Table 3
Reproducibility of the alkaloids ($n = 6$)

Compound	R.S.D. (%)		LOD (ng)
	Intra-day	Inter-day	
1	0.50	1.60	0.16
2	0.75	0.78	0.17
3	0.57	1.70	0.08
4	0.30	1.54	1.32
5	0.54	0.90	0.14
6	0.73	0.93	0.17
7	0.68	1.34	0.15
8	0.72	1.33	0.04
9	0.60	1.61	0.22
10	0.78	1.21	0.17
11+12	0.78	1.14	1.15

each peak was less than 0.46% for six replicated injections.

3.2.3. Linearity

The linearity of the peak-area ratios (y) vs. concentration (x, mg/ml) curve for each of the 12 alkaloids was investigated in the ranges: 0.0003–0.0840 mg/ml for **5**, **6**, **7**, **9**, **10** and **11+12**; 0.0011–0.2940 mg/ml for **1**, **3**, **4** and **8**; 0.0088–0.5962 mg/ml for **2**. Results of the regression analyses and the correlation coefficients (r) are shown in Table 4.

3.2.4. Accuracy

Suitable amounts (0.14–1.78 mg, about 16–43% of the evodia alkaloid contents) of the 12 alkaloids were added to a sample of Evodiae Fructus of known alkaloid content and the mixture was extracted and analyzed using the proposed procedure. The recoveries of the alkaloids were 98.6–101.4% ($n = 3$), as shown in Table 4. All the tailing factors of the peaks are very close to unity.

3.3. Determination of the alkaloids in Evodiae Fructus sample

When the test solution was analysed by HPLC under the selected conditions, the chromatogram shown in Fig. 3B was obtained. By substituting the peak-area ratios of the individual peaks for y in the equations listed in Table 4, the contents of the individual alkaloids in the Evodiae Fructus were obtained: ($n = 3$, mg/g±S.D.) **1**, 9.17±0.04; **2**, 69.14±0.06; **3**, 18.91±0.02; **4**, 12.90±0.06; **5**,

Table 4
Linear ranges, correlation coefficients (r) and recovery studies of the alkaloids

Compound	Linear range (mg/ml)	Slope	Intercept	r	Recovery (%)
1	0.0016–0.1070	30.69	0.01	1.0000	98.77
2	0.0088–0.5962	8.25	0.00	0.9999	100.50
3	0.0039–0.2940	26.46	0.04	0.9999	99.53
4	0.0033–0.2260	36.62	0.64	0.9996	98.60
5	0.0003–0.0197	32.55	0.00	1.0000	101.25
6	0.0009–0.0590	21.35	0.01	1.0000	100.25
7	0.0007–0.0502	30.66	0.01	1.0000	99.74
8	0.0036–0.2435	31.35	0.06	0.9999	101.36
9	0.0011–0.0840	16.20	0.00	1.0000	100.79
10	0.0009–0.0579	30.83	0.00	0.9999	99.55
11+12	0.0004–0.0262	16.84	0.00	0.9999	101.11

1.22±0.02; **6**, 3.77±0.01; **7**, 3.11±0.01; **8**, 14.53±0.01; **9**, 5.34±0.01; **10**, 4.14±0.06; **11+12**, 2.15±0.02; total, 144.38±0.03.

4. Conclusions

This work has successfully demonstrated that by optimizing parameters such as HOAc, NaOAc concentration of the eluent system and the column used, high-resolution separations of a complicated mixture can easily be achieved. The proposed HPLC method is suitable for the determination of alkaloids in the crude extracts of an Evodiae Fructus sample.

Acknowledgments

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