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Simultaneous analysis of protoberberine, indolequinoline and quinolone alkaloids in coptis–evodia herb couple and the Chinese herbal preparations by high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

A new and sensitive method has been developed for analysis of protoberberine, indolequinoline and quinolone alkaloids in coptis–evodia herb couple and the Chinese herbal preparations by using HPLC in combination with electrospray mass spectrometry (ESI-MS). Different LC and cone voltages have been optimized prior to this in order to obtain better results and sensitivity. The method was carried out by using a spherigel C_{18} analytical column with a gradient solvent system of acetate buffer–acetonitrile–methanol. The contents of alkaloids were measured by pseudomolecule ion on the selective ion recording (SIR) mode. Linearity of around three orders in the magnitude of concentration was generally obtained and limits of detection for these compounds were in the range of 9–30 pg.

Keywords: Protoberberine alkaloids; Indolequinoline alkaloids; Quinolone alkaloids; HPLC–ESI-MS

1. Introduction

The traditional Chinese medicine (TCM), most of which are formulations, has been attracting more and more attention for their complementary therapeutic effects to western medicines [1,2]. The herb couples (mixture of two herbs) are much simpler than complicated formulations in composition but retain the basic therapeutic features [3]. Coptis (*Coptidis rhizoma*) has the effects of suppressing fever, dispelling dampness and removing toxicosis, and evodia (*Evodiae fructus*) possesses the actions of warming the middle torso, dispelling cold, causing vitality to descend and controlling pain [4]. The two herbs are the basic components of Chinese herbal preparations such as Zuojin wan, Fanzuojin wan, Ganlusan wan, Zhuyuwan fang, etc. Zuojin wan has the effects of the healing of hypochondric and costal pain, stomach ache, acid regurgitation, nausea and upset according to Chinese medicine theories and is widely adopted for the clinical use in China [5]. Recent pharmacological studies showed that it also had inhibited gastric secretion and pepsin, anti-flammatory, antifungal and analgesic activities [6,7]. However, the remedial mechanism is still not well understood. So far, it is widely accepted that multiple constituents are responsible for the mutual therapeutic effect. This situation makes the quality control of its products very difficult. Thus, the development of a simple and sensitive quality control method for coptis–evodia formula and Chinese herbal preparations is necessary.

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Currently, two strategies of quality control for TCM products are mainly employed. The most widely applied strategy is to determine single or a few mark compounds, which usually are previously identified bioactive constituents, for the assessment of quality [5]. The contents of single or a few mark compounds cannot accurately reflect the quality of TCM products, due to multiple constituents involved in the therapeutic effect. Another strategy is based on the chromatographic fingerprint technology. Comparing with the previous strategy, it substitutes aggregate HPLC peaks of the major ingredients for a few mark compound as the quality indexes and was used to assess the batch-to-batch consistency of TCM products. This strategy has been gradually applied for the quality control standards of more and more TCM products in China. Nevertheless, this strategy is a “blind analysis”, which lacks for the chemical information about the ingredients, so it could not reflect the pharmaceutical activity of the TCM products. To solve this problem, chromatographic fingerprint technology, which could reflect most of active consitituents of TCM products, is necessary.

The pharmacologically active ingredients of *C. rhizoma* are a number of protoberberine alkaloids [8–11], and those of *E. fructus* are indolequinoline and quinolone alkaloids [12–16], as shown in Fig. 1. Several methods have been reported for the determination of some of these protoberberine alkaloids, including thin-layer chromatography (TLC) [17–19], micellar chromatography [20], high-performance liquid chromatography (HPLC) [21–25], high-performance liquid chromatography–electrospray mass spectrometry (HPLC–MS) [26,27], high-speed counter-current chromatography [28], capillary electrophoresis (CE) [29] and capillary electrophoresis-ionspray mass spectrometry (CE-MS) [30,31]. However, HPLC–MS [26,27] analyzed only protoberberine alkaloids of *C. rhizoma*, did not analyze simultaneously protoberberine, indolequinoline and quinolone alkaloids. Several methods have been established to determine some of these indolequinoline and quinolone alkaloids in crude drug such as TLC [32–34], HPLC [35–38], CE [39]. Chu and Sheu [40] developed an HPLC method successfully, and Lee and Sheu [41] developed a CE method for simultaneously assaying the two herbs. However, The solvent systems provided by Chu and Sheu [40] was not suitable for the HPLC–MS analysis owing to the presence of sodium and high concentration salts or sodium dodecyl sulfate. The CE method actually assayed some of 17 alkaloids by micellar electrokinetic capillary chromatography and capillary zone electrophoresis. To our knowledge, these methods could not provide molecular mass and chromatographic fingerprint for the rapid identification of the constituents and quality control in coptis–evodia formula and preparations of Chinese medicine.

HPLC–MS has been proven to be a powerful approach for the rapid identification and determination of the ingredients in botanic extracts and TCM because of its low detection limit, high specificity and molecular mass information. In this paper, an HPLC–ESI-MS method for the qualitative and quantitative analysis of the major alkaloids in coptis–evodia formula and Chinese herbal preparations would be described. The selected ion chromatograms of protoberberine, indolequinoline and quinolone alkaloids were developed also by SIR techniques.

2. Experiment

2.1. Instrumentation

A Waters-2695 Alliance HPLC system (Waters Corporation, Milford, MA, USA) with DAD detection set at 250 and 270 nm was coupled to a Micromass ZQ 2000 electrospray mass spectrometer (Manchester, UK). A spherigel C_{18} analytical column (4.6 mm × 250 mm, 5 μm, Johnsson Dalian, China) was used. A linear gradient elution of A (acetate buffer consisting of 50 mM ammonium acetate and 2% acetic acid, pH = 3.65), B (CH_3CN) and C (CH_3OH) was used. The gradient program is presented in Table 1. The solvent flow rate was $1\,ml\,min^{-1}$ and the column temperature was set at 30 °C. The injection volume was 20 μl. By solvent splitting, 20% eluent was allowed to flow into the mass spectrometry. The ESI-MS spectra were acquired in the positive ion mode. The conditions of HPLC–MS analysis were as follows: desolvation gas, flow rate $250\,l\,h^{-1}$, cone gas $50\,l\,h^{-1}$, desolvation temperature 250 °C. The source temperature 105 °C, capillary voltage 3500 V and cone voltage 35 V, scan range 200–600 u, respectively.

2.2. Reagents and materials

HPLC-grade acetonitrile, methanol, ammonium acetate and acetic acid were obtained from Tedia Company Inc. (Fairfield, OH, USA). Deionized water (Milli-Q water system, Millipore Bedford, MA, USA) was used in the preparation of the samples and buffer solution. Jatrorrhizine hydrochloride, palmatine hydrochloride, berberine hydrochloride, evodiamine, rutaecarpine and tetrahydropalamatine were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *C. rhizome*, *E. fructus*, Zuojin wan, Fan zuojin wan, Ganlusan wan, and Zhuyuwan fang were purchased from the Chinese herbal market and a pharmaceutical company in Changsha, China.

Table 1
Solvent gradient program of HPLC analysis

Time (min)	A (%)	B (%)	C (%)
0	78.0	20.0	2.0
20	65.0	25.0	10.0
22	43.0	27.0	30.0
30	41.0	28.0	31.0
37	18.0	41.5	40.5
47	10.0	45.0	45.0
57	10.0	45.0	45.0

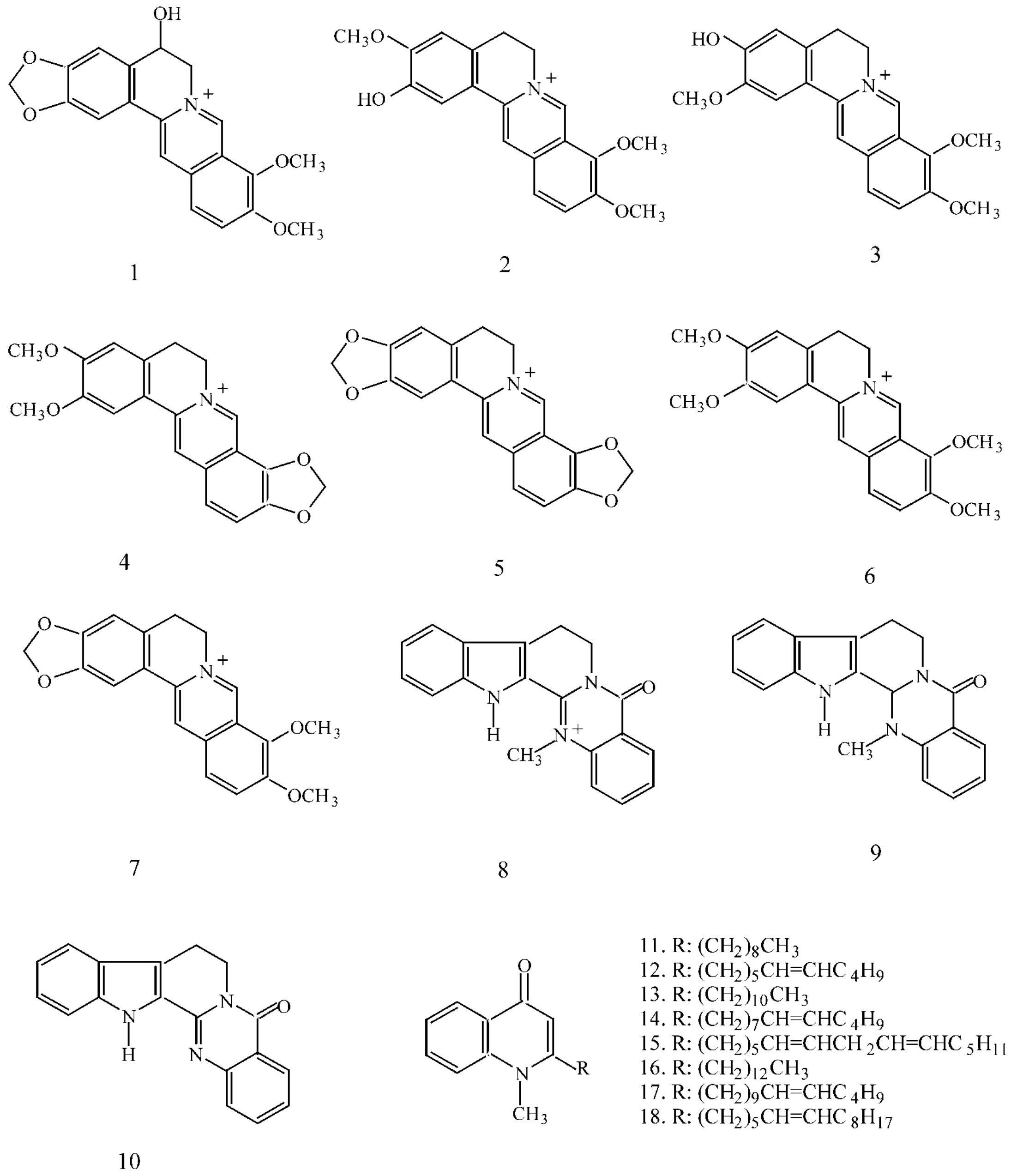

Fig. 1. Structures of the 18 alkaloids: (**1**) berberastine; (**2**) columbamine; (**3**) jatrorrhizine; (**4**) epiberberine; (**5**) coptisine; (**6**) palmatine; (**7**) berberine; (**8**) dehydroevodiamine; (**9**) evodiamine; (**10**) rutaecarpine; (**11**) 1-methyl-2-nonyl-4(1H)-quinolone; (**12**) 1-methyl-2-[(Z)-6-undecenyl]-4(1H)- quinolone; (**13**) 1-methyl-2-undecyl-4(1H)-quinolone; (14) evocarpine; (**15**) 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)-quinolone; (**16**) dihydroevocarpine; (**17**) 1-methyl-2-[(Z)-10-pentadecenyl]-4(1H)-quinolone; (**18**) 1-methyl-2-[(Z)-6-pentadecenyl]-4(1H)- quinolone.

2.3. Preparing the extracts of herb couple, Chinese herbal preparations

A 0.2 g sample of the pulverized herb couple (containing 0.1 g *C. rhizome* and 0.1 g *E. fructus*) or Chinese herbal preparation of Zuojin wan, Fanzuojin wan, Ganlusan wan, and Zhuyuwan fang was extracted with 70% methanol (20 ml) by sonicating for 15 min, then centrifuging for 5 min. Extraction was repeated once. The supernatants were placed in a 50 ml volumetric flask, then diluted to volume with 70% methanol aqueous solution. Then 2 ml solutions were filtered through a 0.45 μm filter into an HPLC vial prior to LC-MS analysis.

2.4. Standard solutions

A mix standard stock solution containing jatrorrhizine hydrochloride (100 μg ml^{-1}), palmatine hydrochloride (90 μg ml^{-1}), berberine hydrochloride (120 μg ml^{-1}), evodiamine (100 μg ml^{-1}), and rutaecarpine (120 μg ml^{-1}) was prepared in methanol. A set of six standard solutions containing between 10.0 and 10 000 ng ml^{-1} of jatrorrhizine hydrochloride, 9.0 and 9000 ng ml^{-1} of palmatine hydrochloride, 12.0 and 12 000 ng ml^{-1} of berberine hydrochloride, 10.0 and 10 000 ng ml^{-1} of evodiamine, 12.0 and 12 000 ng ml^{-1} of rutaecarpine were prepared. The solutions were stored in refrigerator.

3. Results and discussion

3.1. Optimization chromatographic separation conditions

Chu and Sheu [40] reported two distinctly different ion-pair reversed-phase HPLC methods to analyze the two herb drugs successfully. However, the mobiles were not suitable for the LC-MS analysis owing to the presence of sodium, high-concentration salts and sodium dodecyl sulfate (SDS). Modifying the method [40] by eliminating SDS, using ammonium acetate instead of sodium acetate, a gradient solvent system was developed in this experiment.

For optimization the separation conditions, the effects of concentrations of ammonium acetate and acetic acid in the mobile were investigated. When ammonium acetate was not added, the peaks of quaternary alkaloids (**1**–**8**) were very broad and overlapped. At concentrations higher than 30 mM, all alkaloids could be separated, except compounds **3**, **4** and **8**, and compounds **17** and **18**, which overlapped completely. But 60 mM often resulted in stopping flow, 50 mM ammonium acetate should be selected.

With the addition of acetic acid to the mobile phase, higher and narrower peaks for compounds **1**–**8** were achieved. At a concentration of 2%, the most satisfactory resolutions were obtained. To determine the optimal elution conditions for separating the 18 alkaloids, various linear gradients of acetonitrile, methanol and a buffer solution consisting of 50 mM ammonium acetate and 2% acetic acid at a flow rate of 1 ml min^{-1} were employed. Under the conditions given in Table 1, 18 alkaloids in coptis–evodia herb couples could be well separated, except compounds **3**, **4** and **8**, and compounds **17** and **18**, which overlapped completely. However, efforts to try to separate compounds **3**, **4** and **8** by modifying their conditions were found to be invalid. Taking into account selected ion recording (SIR) technique and different molecular ions in compounds **3**, **4** and **8**, the conditions were used for separating the herb couple.

A chromatogram showing the separation of methanol–water extract of *C. rhizoma*, *E. fructus* and Fanzuojin wan, using the method, is shown in Fig. 2.

3.2. Optimization cone voltage conditions

Under the above condition, effect of cone voltage on signal intensity of the mix standard solutions was investigated and it has a tremendous effect as shown in Fig. 3. Fig. 3 shows the results of increasing cone voltage on the intensity of the pseudomolecule ion $[M+H]^+$ or $[M]^+$ of the five mix standard solutions. The intensity of the pseudomolecule ion $[M]^+$ of jatrorrhizine hydrochloride, berberine hydrochloride and $[M+H]^+$ of evodiamine gain the maximum at cone voltage 35 V. The intensity of $[M]^+$ of palmatine hydrochloride and protonated molecules $[M+H]^+$ of rutaecarpine gain the maximum at cone voltage 45 V. At low (15 V) and high (60 V) voltages, the signal-to-noise ratios of the total ion current were deteriorated. In order to better determine protoberberine and indolequinoline alkaloids, an optimum could be reached at cone voltage 35 V.

3.3. Identification of the alkaloids in coptis–evodia herb couples and the Chinese herbal preparations

Table 2 lists the retention times (t_R) and pseudomolecular ion in HPLC peaks. The 18 alkaloids, **1**–**18**, in the samples were identified by comparing the online UV and MS information with authentic standards or literature data [26,35,42]. Peaks **1**–**7** are protoberberine alkaloids with retention order in agreement with the reported HPLC profiles [26]. Peaks **8**–**10** are three indolequinazoline alkaloids, peaks **11**–**18** are quinolone alkaloids with retention order identical to the reports [35,42]. The chromatographic fingerprints of three bio-active components, protoberberine alkaloids, indolequinoline and quinolone alkaloids in Fanzuojin wan were shown in Fig. 4, respectively. The method is better

Table 2
Assignment of peaks in Figs. 2 and 4

Compound	t_R[a] (min)	m/z $[M]^+$	m/z $[M+H]^+$	Compound	t_R[a] (min)	m/z $[M]^+$	m/z $[M+H]^+$
1	11.24	322.2[b]	–	**10**	35.95	–	288.4
2	15.26	338.3	–	**11**	41.79	–	286.5
3	15.83	338.3	–	**12**	42.89	–	312.5
4	16.12	336.2	–	**13**	46.25	–	314.5
5	17.09	320.2	–	**14**	47.10	–	340.5
6	21.63	352.3	–	**15**	48.34	–	366.5
7	22.56	336.2	–	**16**	51.53	–	342.6
8	16.20	302.4	–	**17**/**18**	52.15	–	368.5
9	32.50	–	304.4				

[a] Retention time of SIR.

[b] $[M\text{-}HCHO]^+$.

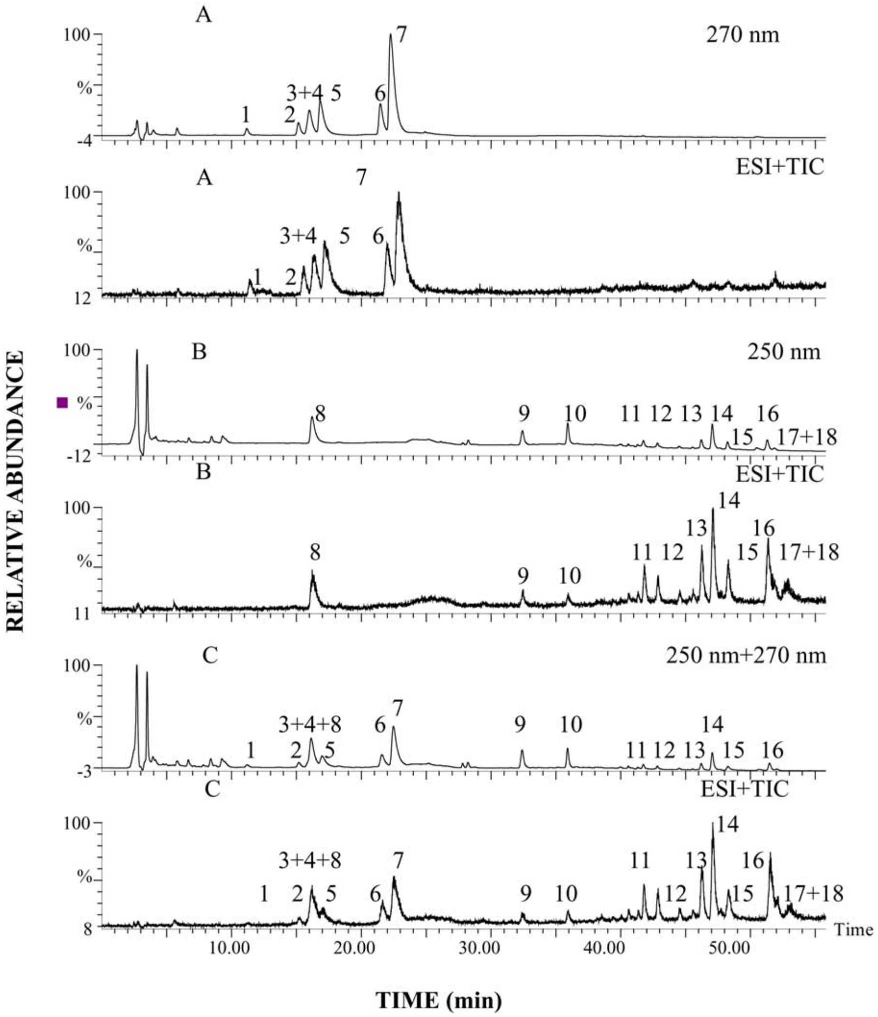

Fig. 2. HPLC–UV–ESI-MS-total ion current (TIC) chromatograms for the extract of *C. rhizoma* (A), *E. fructus* (B) and Fanzuojin wan (C), with a 1:5 post-column stream splitting. Chromatographic conditions are described in Section 2.1. The peak symbols are as in Fig. 1.

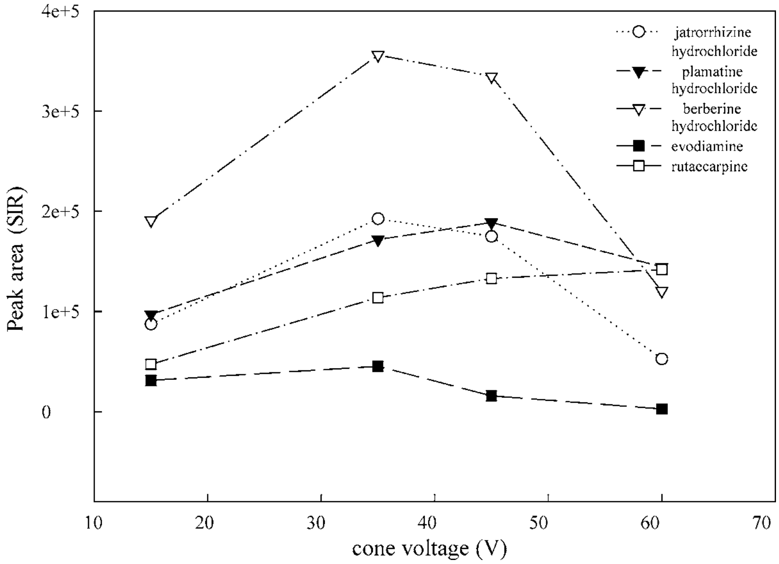

Fig. 3. The effect of cone voltage on SIR signal intensity of five mix standards.

than previously published techniques in identifying the constituents and quality control in coptis–evodia herb couple and the Chinese herbal preparations.

In addition, SIR was investigated in our experiments. The SIR technique was more sensitive than full scanning. The SIR techniques reflected better chromatographic fingerprints of three bio-active components, protoberberine alkaloids, indolequinoline and quinolone alkaloids than full scanning. Fig. 4(a) shows the SIR chromatograms of *m*/*z* 352.3, 320.2, 336.2, 338.3 and 322.2 for protoberberine alkaloids in the

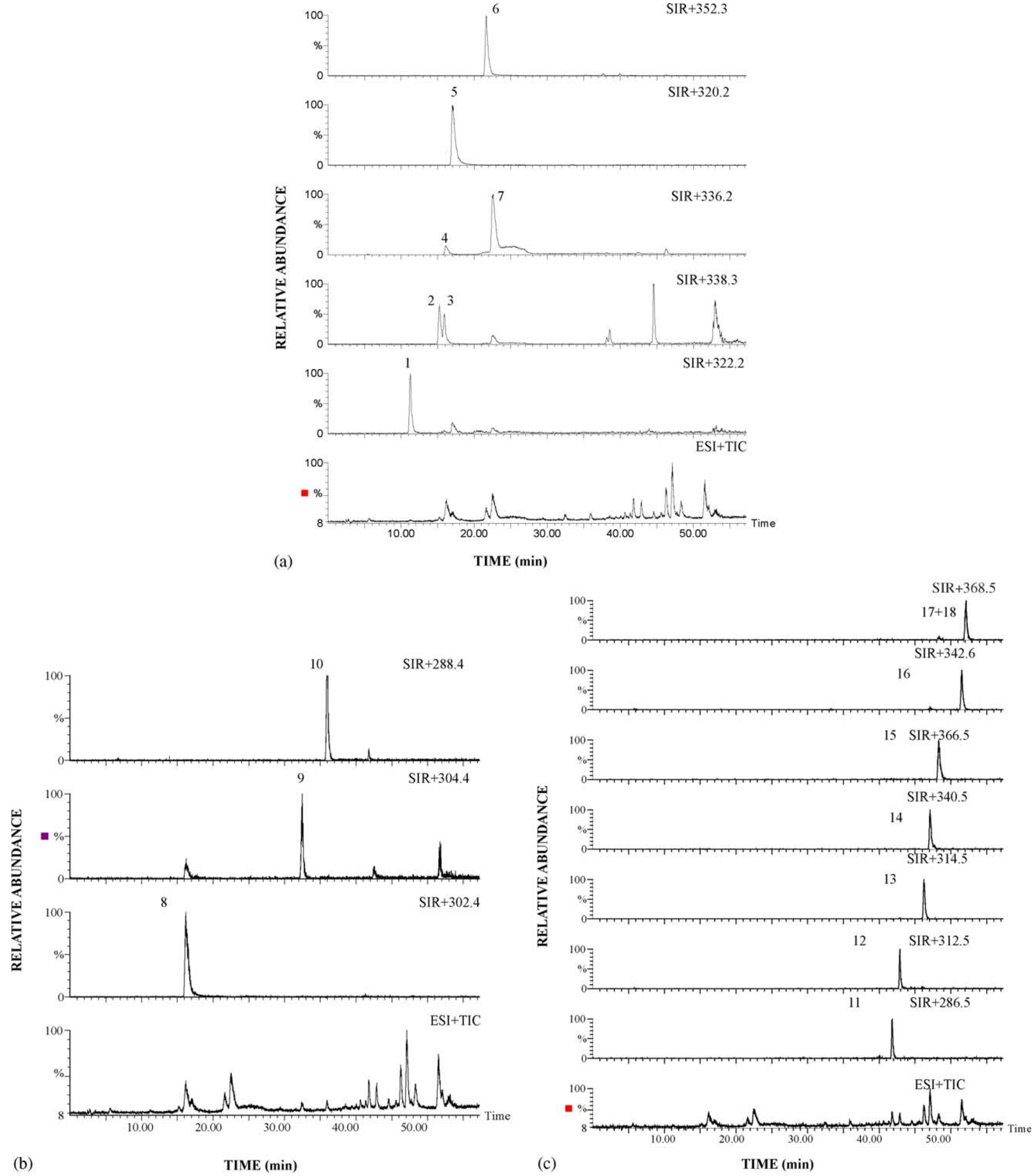

Fig. 4. (a) HPLC–ESI-MS-TIC and HPLC–MS-SIR chromatograms for protoberberine alkaloids in extract of Fanzuojin wan, with a 1:5 post-column stream splitting. Other conditions are the same as in Fig. 2. (b) HPLC–ESI-MS-TIC and HPLC–MS-SIR chromatograms for indolequinazoline alkaloids in extract of Fanzuojin wan, with a 1:5 post-column stream splitting. Other conditions are the same as in Fig. 2. (c) HPLC–ESI-MS-TIC and HPLC–MS-SIR chromatograms for quinolone alkaloids in extract of Fan Zuojin wan, with a 1:5 post-column stream splitting. Other conditions are the same as in Fig. 2.

Table 3
Linear ranges, correlation coefficients (*r*), limits of detection and recovery studies of the alkaloids

Compound	Linear range (ng ml^{-1})	*r*	Recovery (%)	LOD (pg)
3	10–10000	0.9988	99.53	10
6	9–9000	0.9983	98.79	9
7	12–12000	0.9995	100.30	24
9	20–10000	0.9978	101.10	30
10	12–12000	0.9971	100.25	24

Table 4
Contents (mg g^{-1}) of the five alkaloids in the herb couple and the Chinese herbal preparations (mean ± S.D.; *n* = 3)

Compound	Herb-couple	Zuojin wan	Fanzuojin wan	Ganlu san	Zhuyuwan fang
3	1.86 ± 0.06	2.36 ± 0.08	0.55 ± 0.05	2.49 ± 0.08	1.76 ± 0.05
6	9.35 ± 0.04	16.00 ± 0.12	2.66 ± 0.10	12.53 ± 0.11	9.05 ± 0.10
7	32.23 ± 0.12	55.07 ± 0.21	8.10 ± 0.18	43.19 ± 0.15	30.32 ± 0.20
9	1.68 ± 0.02	0.34 ± 0.06	2.59 ± 0.04	1.14 ± 0.08	1.61 ± 0.03
10	2.28 ± 0.03	0.81 ± 0.07	3.28 ± 0.05	1.55 ± 0.06	2.13 ± 0.05

positive ion mode, Fig. 4(b) shows the SIR chromatograms of *m*/*z* 288.4, 304.4, and 302.4 for indolequinolone alkaloids in the positive ion mode, and Fig. 4(c) shows the SIR chromatograms of *m*/*z* 368.5, 342.6, 366.5, 340.5, 314.5, 312.5, and 286.5 for quinolone alkaloids in the positive ion mode. Obviously, the signal-to-noise ratio value indicates that the SIR mode offered a better detection limit than the full scanning mode. So in our experiments, *m*/*z* 338.3, 352.3, 336.2, 304.4, and 288.4 were used as qualifier ions for 3, **6**, **7**, **9** and **10**.

3.4. Validation of method

3.4.1. Precision

The repeatability (relative standard deviation) of the proposed method in terms of the peak-area in five replicate injections were 1.67, 2.87, 4.56, 2.33, 1.92, 3.12, 2.51, 4.35, 2.45, 1.51, 2.12, 3.47, 2.53, 2.16, 2.34, 2.07, 2.98% (intra-day) for compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **14**, **15**, **16**, **17**/**18**, respectively, and 2.79, 3.12, 4.68, 2.89, 2.93, 3.45, 3.18, 4.47, 3.15, 2.83, 3.10, 3.59, 2.97, 3.05, 3.45, 3.34, 3.65% (inter-day) for compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **14**, **15**, **16**, **17**/**18**, respectively; The detection limits (S/N = 3) for the five alkaloids were 9–30 pg.

3.4.2. Linearity

The linearity calibration curves were made on at least six experiments of each reference compound. The regression equation was calculated in the form of $Y\psi\Im \leftarrow AXarrowB$ where *Y* and *X* were the values of the peak-areas and sample concentration, respectively. The results and the correlation coefficients (*r*) are listed in Table 3.

3.4.3. Accuracy

The recoveries of the alkaloids were determined by the standard addition method. The compounds **3** (5.0 μg), **6** (4.5 μg), **7** (6.0 μg), **9** (5.0 μg) and **10** (6.0 μg) were spiked into the sample and extracted as described above. For comparison, an unspiked sample was concurrently prepared and analysed. The recoveries of the alkaloids were 98.79–101.10% (*n* = 3), as shown in Table 3.

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

Analysis of herb couple and Chinese herbal preparation with HPLC–ESI-MS gave the results shown in Table 4. Data in Table 4 show that the five alkaloids contents are quite different in four Chinese herbal preparations which have different therapeutic effects. However, other 13 alkaloid contents were not determined. From the results, multiple constituents are responsible for the therapeutic effects of Chinese herbal preparations, and the contents of ingredients may also affect therapeutic effect of Chinese herbal preparations.

4. Conclusion

In this paper, protoberberine, indolequinoline and quinolone alkaloids in coptis–evodia herb couple and the Chinese herb preparations were analyzed using HPLC–ESI-MS; high sensitivity and selectivity have been achieved with MS as the detectors. The unity of HPLC–ESI-MS provides more qualitative and quantitative information comparing with general HPLC. And complex extraction or sample pretreatment is unnecessary. This study provided the chemical support for the bioactive chromatographic fingerprint technology and facilitates to improve the quality of commercial Chinese herbal preparations containing the coptis–evodia herb couple and also for quality control in pharmaceutical factories.

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