Simultaneous Determination of Six Quaternary Ammonium Alkaloids in *Coptidis Rhizoma* by UPLC

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Abstract: **Objective** To establish a new, rapid, and reliable reversed-phase ultra performance liquid chromatography (RP-UPLC) method for the simultaneous determination of six quaternary ammonium alkaloids (QAAs) in *Coptidis Rhizoma*. **Methods** The effect of different experimental parameters on the analysis of QAAs by RP-UPLC was evaluated. **Results** Optimal resolution was achieved with an Acquity UPLC BEH C_{18} column using a gradient elution profile and a mobile phase consisting of water spiked with 10 mmol/L ammonium bicarbonate (A, pH adjusted to 10.0 by ammonia water) and acetonitrile (B), at a flow rate of 0.30 mL/min and wavelength of 345 nm. The column temperature was set at 30 ℃. The proposed method was found to be reproducible, precise, and rapid according to the method validation. **Conclusion** The proposed method, which is compatible with MS analysis and the preparation of QAA, provides some helpful insights into the quality control of *Coptidis Rhizoma*.

Key words: *Coptidis Rhizoma*; method validation; quaternary ammonium alkaloids; simultaneous determination; ultra performance liquid chromatography
DOI: 10.3969/j.issn.1674-6384.2012.02.011

Introduction

Alkaloids have attracted increasing interest around the world for their significant bioactivity and have been widely applied in central nervous system diseases (Kulkarni and Dhir, 2007; 2010; Ye *et al*, 2009) and endocrine system diseases (Gu *et al*, 2010). They also exhibit antibacterial, antivirus (Bing *et al*, 2009; Chen *et al*, 2008a), and anticancer (Wang *et al*, 2009; Deng *et al*, 2006) activities. In the study of natural products, alkaloids are very important resources and have considerable potential in wide applications.

Alkaloids often have similar chemical structures, especially quaternary ammonium alkaloids (QAAs) isolated from *Coptidis Rhizoma*. Nevertheless, with the structure of an isoquinoline mother nucleus (Fig. 1), QAAs share similar physical and chemical properties (strong basicity and polarity), which leads to difficulty in separation. This is a significant challenge in the analysis of QAAs. Therefore, it is necessary to develop a rapid, sensitive, and reliable qualitative and quantitative analysis method for QAAs.

Until now, several analytical methods have been employed to separate QAAs in *Coptidis Rhizoma*, including HPLC (Pharmacopeia Committee of P. R. China, 2010; Geng *et al*, 2010), HPLC-MS (Ren *et al*, 2007; Ding *et al*, 2007), high-speed counter-current chromatography (HSCCC) (Zhang, Wang, and Wang, 2011) and capillary electrophoresis (CE) (Chen *et al*, 2008b; Stöckigt *et al*, 2002). However, they have shortcomings, e.g., HPLC is time-consuming (Chu and Sheu, 1996) and the additive sodium dodecyl sulphate (SDS) is not suitable for mass spectrometry (MS)

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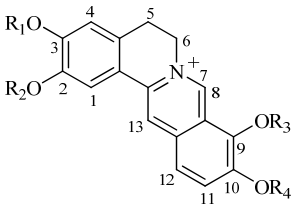
Received: September 15, 2011; Revised: December 10, 2011; Accepted: February 28, 2012
Fund: National Natural Science Foundation of China (30873385, 81173542), the Beijing Natural Science Foundation (7112128), and the Open Research Fund of State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicinal Resources



jatrorrhizine (JAT) R_1=H R_2=R_3=R_4=CH_3
columbamine (COL) R_1=R_3=R_4=CH_3 R_2=H
coptisine (COP) R_1=R_2=R_3=R_4=-CH_2-
epiberberine (EPI) R_1=R_2=CH_3 R_3=R_4=-CH_2-
palmatine (PAL) R_1=R_2=R_3=R_4=CH_3
berberine (BER) R_1=R_2=-CH_2- R_3=R_4=CH_3

Fig. 1 Chemical structures of six alkaloids

(Wang *et al*, 2008; Pharmacopeia Committee of P. R. China, 2010). The established UPLC conditions were poor in baseline separation and reproducibility (Chen *et al*, 2008a; Gao, Yang, and Marriott, 2010).

Here we developed a specific, rapid, and reliable UPLC method suitable for simultaneous quantification of six alkaloids without non-volatile ion-pair reagents, which improves the preparation and quality control of effective components in *Coptidis Rhizoma* and gives some useful insights into the analysis of other types of QAA.

Materials and methods

Chemicals and instrument

Coptis chinensis Franch. was obtained from Hubei Province, China and was identified by Prof. XIAO Xiao-he. Berberine hydrochloride, jateorrhizine hydrochloride, palmatine hydrochloride, coptisine, epiberberine, and columbamine were purchased from National Institute for Food and Drug Control (Beijing, China). Acetonitrile and methanol of HPLC grade were from Fisher Chemicals (Pittsburg, USA). Other chemicals of analytical grade were purchased from Beijing Chemical Factory (Beijing, China). Water was purified by using a Milli-Q water purification system (Millipore, USA).

The analysis of alkaloids from *Coptidis Rhizoma* was carried out on a Waters Acquity UPLC™ system (Waters, USA), including binary solvent delivery pump, auto sampler manager, column compartment, and photo diode array (PDA) detector, connected to MassLynx 4.1 software. An Acquity UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.8 μm) (Waters Corporation, Australia) was used for separation investigations.

Preparation of standard solution

Reference substances of each alkaloid were accurately weighed and dissolved in 10 mL volumetric flasks with methanol as stock solution at final concentration of 0.16, 0.20, 0.40, 0.44, 0.60, and 0.84 mg/mL for JAT, COL, EPI, COP, PAL, and BER, respectively.

Sample preparation

Coptidis Rhizoma was crushed into powder and 50.00 g was placed into a 1 000 mL flask with precisely 300 mL of purified water. After 0.5 h immersion, the mixture was refluxed thrice, 1.5 h for each time. The extract was filtered while it was hot. Then the extract was transferred into a 10 mL volumetric flask which was made up to its volume with methanol and filtered through a 0.22 μm nylon filter membrane prior to injection into the UPLC system.

Method validation

The proposed analytical method was validated according to international guidelines with respect to linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, and repeatability.

Linearity

Stock solutions containing six reference compounds were prepared and diluted into appropriate concentration for construction of calibration curves. At least six levels of the solution concentration were analyzed in duplicates and then calibration curves were constructed by plotting the peak area against the concentration of each analyte. The acquired regression equation was calculated in the form of $Y = aX + b$, where Y and X were the peak area and concentration of the reference compound, respectively.

LOD and LOQ

The stock solutions containing six reference compounds were diluted to a series of appropriate concentration with methanol and aliquots of the diluted solutions were injected into UPLC for analysis. The LOD and LOQ under the chromatographic conditions used were determined at signal-noise (S/N) ratio of approximate 3 and 10, respectively.

Precision

Intra- and inter-day variations were utilized to determine the precision of the proposed method. The known concentration of six standard solutions was tested. For the intra-day variability test, six replicates of the mixed standard solutions were analyzed within one day, while for the inter-day variability test, the solutions

were examined in duplicates for consecutive 3 d. Variations were expressed by relative standard deviation (RSD).

Accuracy

The recovery test was used to evaluate the accuracy of the assay. A known amount of standards were added into a certain amount of sample. The mixture was treated and analyzed using the method described above. Five replicates of the test were performed.

Repeatability

To confirm repeatability, five replicates of the same samples were extracted and analyzed as described above. The RSD value was calculated as a measurement of method repeatability.

Results and discussion

Optimization of UPLC conditions

Feasibility was evaluated upon different solvent systems, such as acetonitrile-water and methanol-water mixtures at various ingredients, at distinct flow rates (0.1－0.5 mL/min), at a range of pH values (3.0－10.0), and at a series of column oven temperatures (25－50) ℃. The best resolution of QAAs was achieved under the following conditions: acetonitrile-water (10 mmol/L ammonium bicarbonate, pH 10.0) as the mobile phase, a flow rate of 0.3 mL/min, and a temperature of 30 ℃.

Since QAAs are basic compounds, their retention time varied considerably with the mobile phase at pH values within the range of 3.0－10.0. As shown in Figs. 2 and 3, when the pH values were increased from 3.0 to 7.0, JAT, COL, and COP were unresolved. When the pH value reached 8.0, the peak order of JAT and COL reversed. As the pH value increased continuously, QAAs were gradually separated and the selectivity index α increased by degrees (Fig. 4). Finally, the six alkaloids were separated completely at pH 10.0. Therefore, pH 10.0 was chosen as the optimal pH value because of the reasonable retention time and resolution.

The phenomenon above suggests that under acidic conditions, the protonated alkaloids could be eluted and failed in complete separation. It seemed that low pH value had little influence on the separation of QAAs. On the contrary, at alkaline conditions, these QAAs could be successfully separated, because when the pH value of the mobile phase approached the p*K*a of a compound, its retention time did significantly vary with the pH value. In general, this occurred in the pH range of the compounds' p*K*a ± 1.5 (Snyder *et al*, 1997).

During the whole process, the retention behavior of JAT and COL changed remarkably, while retention time and resolution of other QAAs kept almost invariable. The reason for this phenomenon may be that unlike other QAAs, JAT and COL have the group of phenolic hydroxyl which will present different forms in different pH environments. Meanwhile, their hydrophilicity changed as the pH value was adjusted. Therefore, the

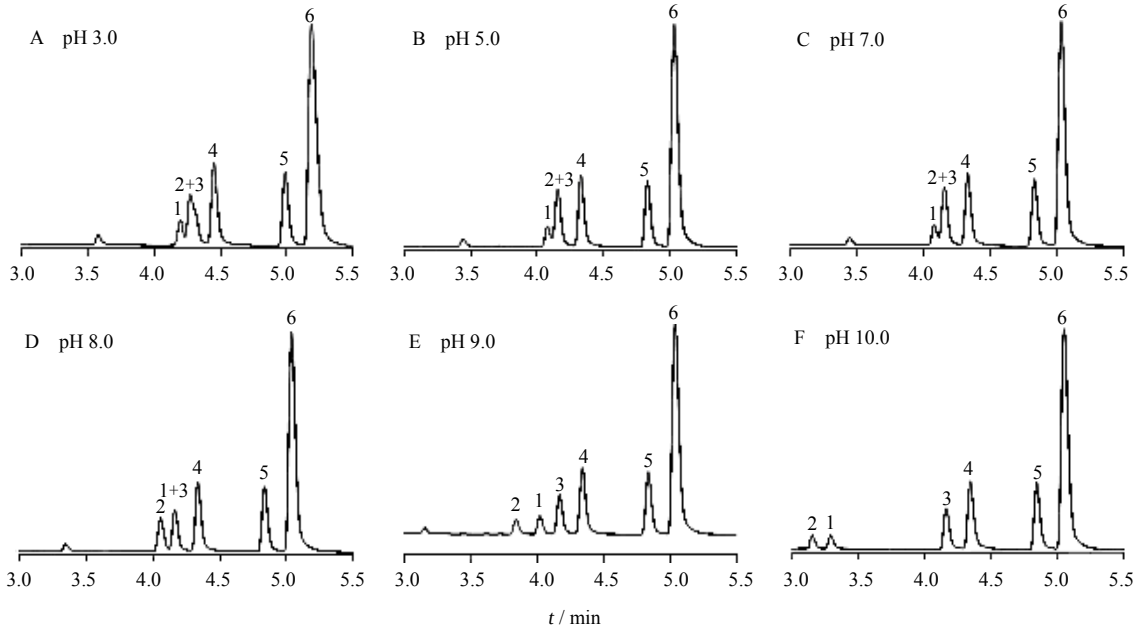


Fig. 2 UPLC profiles of alkaloids with mobile phase at different pH values

Peaks 1－6 are JAT, COL, COP, EPI, PAL, and BER, respectively

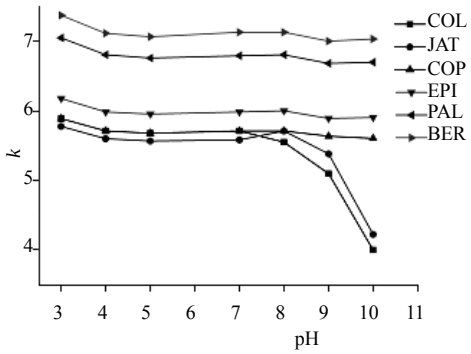


Fig. 3 Effect of mobile phase pH values on capacity factor *k*

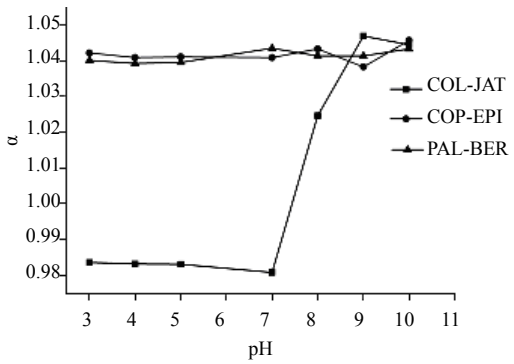


Fig. 4 Effect of mobile phase pH values on selectivity α

retention behaviors of these two components were influenced apparently. To be specific, under basic condition, their phenolic hydroxyl groups were in the form of oxygen anions, thus the hydrophilicity was reinforced, capacity factor *k* decreased and the resolution was ameliorated.

Additionally, it seemed that low pH value had little influence on the separation of QAAs. On the contrary, at alkaline conditions, these QAAs could be successfully separated. The reason may be that when the pH value of the mobile phase approached the p*K*a of a compound, its retention time did significantly vary with the pH value. In general, this occurred in the pH range of the compounds' p*K*a ± 1.5 (Snyder *et al*, 1997).

In addition, buffer salt was added into the mobile phase in order to achieve better separation of these compounds. The following three buffer systems including ammonium formate, ammonium acetate, and ammonium bicarbonate at a series of concentration from 5 to 20 mmol/L, were tested respectively, but no remarkable amelioration was observed. Eventually, ammonium bicarbonate was chosen for its greater buffering capacity (Snyder *et al*, 1997). An excessive amount of buffer could crystallize in the organic phase and resulted in problems regarding pump maintenance (McCalley, 2002), hence the concentration of 10 mmol/L appeared to be satisfactory for our purposes.

When varying the pH value for the purpose of improving selectivity, it may be necessary to simultaneously change the solvent strength to maintain a satisfactory range of *k* (Snyder *et al*, 1997). Because of their similar chemical structures, these six compounds could not be separated simply by isocratic elution. The six peaks were completely separated under the gradient condition: acetonitrile (A) with 0—3 min from 10% to 20% A, and 3—7 min from 20% to 45% A, with good resolution and reasonable running time.

The effect of temperature on retention of the analysis was also evaluated from 20 to 50 ℃ to ensure satisfactory resolution and rapid analysis. When the column temperature increased from 20 to 40 ℃, the retention dropped off linearly, while the resolution of JAT and COL increased. As the temperature rose from 30 to 50 ℃, retention of EPI and COP declined, with column pressure conspicuously decreased. Hence, a column temperature of 30 ℃ was preferred.

In addition, the ultraviolet absorption wavelength of the alkaloids was screened. First, in the range of 200—400 nm for whole wavelength scanning, strong absorption peaks were observed at 250, 270, and 345 nm. The peak purity of target components in these samples was verified using the PDA detector. At 345 nm, most substances did not absorb light and there were fewer interference peaks, with stronger signals and a smoother baseline.

We also found that the retention of QAAs was closely related to their molecular structures, as reflected by different *k* values. For example, COL and JAT contained the hydroxyl group, so *k* decreased. Zou, Zhang, and Lu (2001) proposed that, under certain conditions, the retention behavior and solvation parameters of the solute have a certain relationship. The specific mechanism is not completely understood, so further research is required.

Method validation

The linearity, regression, and linear ranges of the six alkaloids were determined by the proposed UPLC method (Table 1). The correlation coefficient values ($r > 0.99$) indicated appropriate correlations between the investigated compound concentration and the corresponding peak areas within the test ranges. The LOD and LOQ were less than 0.80 and 2.53 μg/mL (Table 1), and the overall intra- and

inter-day variations (RSD) of the six compounds were less than 1.12% and 1.96%, respectively (Table 2). The recoveries ranged from 97.39% to 100.65% (RSD < 4.00%), and overall repeatability RSD was less than 3.00% (Table 2), which indicates the developed method has good accuracy and repeatability.

Table 1 Regression data, LODs, and LOQs of six QAAs

Constituents	Regressive equation	*r*	Linear range / (mg·mL^{-1})	LOQ / (mg·mL^{-1})	LOD / (mg·mL^{-1})
JAT	$Y = 22\,615X + 24.579$	0.9986	0.004－0.160	1.21	0.44
COL	$Y = 13\,981X + 33.517$	0.9989	0.005－0.200	1.52	0.51
COP	$Y = 18\,173X + 41.991$	0.9998	0.011－0.440	2.53	0.80
EPI	$Y = 36\,588X - 134.45$	0.9998	0.010－0.400	2.02	0.58
PAL	$Y = 20\,524X + 227.69$	0.9959	0.015－0.600	1.20	0.44
BER	$Y = 60\,573X + 297.06$	0.9996	0.036－0.840	0.91	0.29

Table 2 Precision, recovery, and repeatability of six QAAs

Constituents	Precision / % (RSD, *n* = 6)		Recovery / % (*n* = 5)		Repeatability / % (RSD, *n* = 5)
	Intra-day	Inter-day	Mean	RSD	
JAT	0.67	1.55	98.73	3.49	1.95
COL	1.03	1.69	99.60	3.58	2.51
COP	0.85	1.78	100.63	3.93	1.89
EPI	1.12	1.84	97.39	3.65	1.85
PAL	0.74	1.79	100.09	2.94	2.21
BER	0.98	1.96	98.37	2.79	2.13

Application

The developed method was applied to the simultaneous separation of the six QAAs from five batches of *Coptidis Rhizoma* which were purchased from Hubei Province. Target components were identified by comparing the retention time and UV spectra with those in the chromatogram of the mixed standard solution. A representative chromatogram of the samples is depicted in Fig. 5. Six QAAs were completely and rapidly separated and the results were presented in Table 3. As shown in Table 3, the total QAA contents of five batches of *Coptidis Rhizoma* closely coincided. The contents of six QAAs in different batches were quite variable, which influences the stability of the quality. Therefore, it is recommended that contents of the individual active components are controlled.

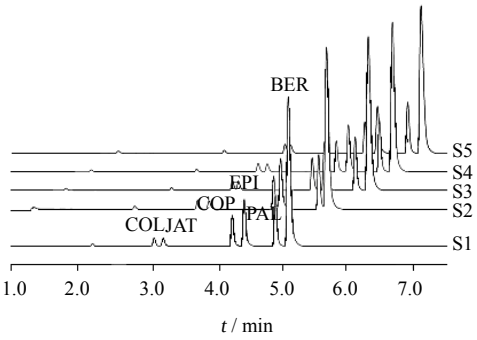


Fig. 5 UPLC chromatograms of six QAAs from *Coptidis Rhizoma*

Conclusion

In the present study, a specific, rapid, and reliable UPLC method was successfully developed for simultaneous separation of six types of QAAs from *Coptidis Rhizoma*. The influence of pH value (3.0－10.0), eluant proportion, buffer system, and column temperature were evaluated.

Table 3 Determination of six QAAs in *Coptidis Rhizoma* ($\bar{X} \pm s$, *n* = 5)

Samples	COL / %	JAT / %	COP / %	EPI / %	PAL / %	BER / %	Total / %
S1	0.86 ± 0.02	0.73 ± 0.12	1.79 ± 0.04	2.63 ± 0.12	2.06 ± 0.05	8.43 ± 0.22	16.50 ± 0.24
S2	0.87 ± 0.01	0.83 ± 0.03	1.63 ± 0.10	2.84 ± 0.03	2.99 ± 0.13	7.98 ± 0.32	17.14 ± 0.35
S3	0.79 ± 0.14	0.82 ± 0.04	1.67 ± 0.07	2.69 ± 0.07	2.27 ± 0.04	8.13 ± 0.05	16.37 ± 0.18
S4	0.89 ± 0.10	0.66 ± 0.08	1.86 ± 0.06	2.59 ± 0.05	2.33 ± 0.08	8.21 ± 0.10	16.54 ± 0.15
S5	0.81 ± 0.13	0.75 ± 0.11	1.82 ± 0.11	2.68 ± 0.08	2.24 ± 0.32	8.24 ± 0.09	16.54 ± 0.16

All the analytes were well separated with less than 7.0 min using a very common column. Most importantly, because it employed a non-volatile buffer, the method could be applied directly to MS detection, which in any case largely obviates the dependence on ion-pair reagents.

Furthermore, the optimization process led to two main conclusions. First, the adjustment of pH value is often the most effective way to ameliorate selectivity for the separation of ionic compounds. In general, this occurred in the pH range of the compounds' p*K*a ± 1.5. Adding of certain concentration of buffer salt will contribute to stability of the pH value. Second, for alkaloids with the same skeleton, gradient elution is more effective than isocratic elution. In addition, when ion-pair reagents were used in QAA separation, alkaloid retention was enhanced and tailing improved but there were some disadvantages, such as strong adsorption, slow column equilibration, and poor reproducibility.

In summary, these conclusions may provide some useful insights into the separation of QAAs from other medicinal plants. The described method is stable so it could be applied to routine quality assessment for this herb. This method may also be used for the analysis and preparation of alkaloid drugs in pharmaceutical preparations and routine laboratory analysis with slight modifications in the extraction procedure.

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