Simultaneous Determination of Cephaeline and Emetine in Ipecac and Its Preparations Using RP-HPLC

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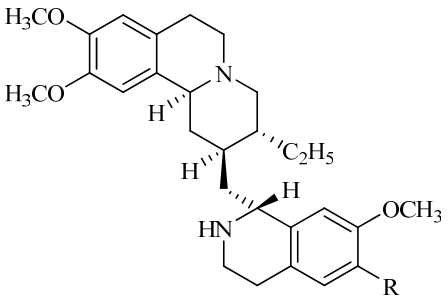
Abstract: **Objective** To control the quality of ipecac and its preparations, and to investigate the simultaneous quantitative determination of cephaeline and emetine. **Methods** After ultrasonic extraction with acidic methanol solution or direct diluting preparations, cephaeline hydrochloride and emetine hydrochloride in ipecac and its preparations were separated within 20 min using a mixture of acetonitrile-methanol-0.1% phosphoric acid (9:3:88) as the mobile phase on a C_{18} column by HPLC. UV detector was set at 205 nm. The flow rate was set at 1.0 mL/min. **Results** The methodological study showed that a good linear correlation existed in the range of 0.014 56—0.2184 μg (r = 0.999 97) for cephaeline hydrochloride and 0.0321—0.321 μg (r = 0.999 97) for emetine hydrochloride, respectively. The average recovery of cephaeline hydrochloride and emetine hydrochloride was 96.93% and 99.47%, and the RSD values (n = 9) were 1.31% and 2.02%, respectively. **Conclusion** The assay is sensitive, accurate, specific, and applicable to comprehensive evaluation on the quality of ipecac and its preparations.

Key words: cephaeline hydrochloride; emetine hydrochloride; ipecac; ipecac preparations; RP-HPLC
DOI: 10.1016/S1674-6384(13)60042-8

Introduction

Ipecac consists of the dried rhizomes and roots of *Cephaelis acuminata* Karsten or *C. ipecacuanha* (Brotero) A. Richard (Rubiaceae) which are mostly imported from Brazil, Costa Rica, and India to China. Ipecac and its preparations are employed as the expectorant and antitussive medicine in many countries and described in *United States Pharmacopoeia* (USP) (The United States Pharmacopoeia Convention, 2012), *European Pharmacopoeia* (EP) (European Directorate for Quality Medicines, 2011), and *Japanese Pharmacopoeia* (JP) (Ministry of Health, Labour and Welfare, 2012). According to the above references, the principal constituents in ipecac roots are emetine, a non-phenolic alkaloid, and cephaeline, a phenolic alkaloid, and the total content of the two alkaloids accounts for more than 84% of total alkaloids (Fig. 1). In USP and EP, the content of the total alkaloids was determined by acid-base titration method. Moreover, in USP, the assay for emetine and cephaeline was recorded. The procedure was tedious and time-consuming, the emetine and cephaeline were eluted and separated respectively through four different columns by using different eluting solvents, then the A values were measured respectively at the wavelength of 283 and 350 nm, and the content was calculated with the difference



cephaeline: R = OH emetine: R = OCH_3

Fig. 1 Chemical structures of cephaeline and emetine

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Received: April 7, 2013; Revised: May 7, 2013; Accepted: July 17, 2013

of *A* at 283 and 350 nm. For the content determination of a batch of samples with the above procedure, the processing time lasts for about 12—16 h for a high level of skilled personnel, and it is difficult to ensure good reproducibility and accuracy.

Ipecac and its preparations were not included in *Pharmacopoeia of People's Republic of China*. Recently, after they were drafted by the import manufacturers and reviewed by National Institute for Food and Drug Control (NIFDC), ipecac crude drug registration standards (SFDA, 2009) were developed in China. The content of total alkaloids is still detected using acid-base titration after organic solvent extraction, which is similar to USP. Due to the flaws in the preparation of test solutions, the accuracy was reduced. This would be discussed in the paper.

Up to now, several analytical methods have been published including HPLC. In the previous studies, Sahu and Mahato (1982) detected emetine and cephaeline in ipecac roots using μPorasil column (silica gel column) with normal phase HPLC. The chromatography did not reach the baseline separation. Bannister *et al* (1979) detected emetine using ion-pair reversed-phase (RP) HPLC with fluorescence detector. In order to improve the sensitivity, emetine was reacted with mercuric acetate and converted into a fluorescent product, but accompanied by environmental pollution. Recently, Asano *et al* (2001) analyzed cephaeline and emetine in human plasma and urine using HPLC method with fluorescence detection. Although the method had higher sensitivity, the pretreatment method was more suitable for the analysis of biological samples.

The content of cephaeline and emetine in commercial crude drugs varies with species, seasons, horticulture conditions, etc. Therefore, it is important to establish a simple and reliable analytical method for the simultaneous determination of cephaeline and emetine in ipecac and its preparations using RP-HPLC. For this reason, the quantitative determination of emetine and cephaeline in ipecac, ipecac extract, and ipecac liquid extract was developed. In addition, in the view of the content of total alkaloids, the contents of cephaeline and emetine determined by titration method do not conform to the contents of cephaeline and emetine determined by HPLC method. The influencing factors of sample pretreatment were discussed and analyzed.

Materials and methods

Reagents and materials

Emetine hydrochloride (purity > 99.5%, batch No. 061M1826V) was purchased from Sigma; Cephaeline hydrochloride (purity > 97.8%, batch No. 201201) was prepared by our laboratory.

Ipecac crude drug, ipecac crude drug (Costa Rica), ipecac crude drug (Brazil), and ipecac crude drug (India), were obtained from Anguo in Hebei province (China), and identified by Prof. DUAN Ji-ping in Hebei Institute for Food and Drug Control. Ipecac extract (100 mL/bottle, batch No. 20120101), ipecac liquid extracts (100 mL/bottle, batch No. 20120102, 20120103, and 20120104), and ipecac tincture (100 mL/bottle, batch No. 20120105) were provided by Hebei Ruijingkang Biotechnology Co., Ltd. (China).

Methanol, acetonitrile, and phosphoric acid used for the mobile phase were of HPLC grade from Fisher Scientific (USA). Purified water was used from a Milli-Q system (Millipore, USA). Other reagents were all of analytical grade.

Instruments

The chromatography system consisted of Shimadzu LC—20AT High-pressure Pump, SPD—M20A Diode Array Detector, SIL—20A Auto-injector, and LC-solution Chromatography Workstations (Shimadzu, Japan). Different columns such as Shimadzu ODS-VP column, Dikma diamonsil C_{18} column, and Shimadzu ODS-SP column were used for chromatography system.

Sample preparation

The powdered ipecac (0.1 g) was placed to a stopper conical flask, weighed accurately, added accurately with 25 mL of hydrochloride-60% methanol (1:200), stoppered and weighed, and extracted with ultrasonic for 30 min (power of 250 W and frequency of 40 kHz). After being cooled to room temperature and weighed again, the loss of weight was replenished with methanol. The sample was mixed well and filtered. A quantity of ipecac preparations equivalent to about 1 g crude drug was taken to a 25 mL volumetric flask, added with 60% methanol to scale, mixed well and filtered. The ipecac successive filtrate (2 mL) or preparations successive filtrate (1 mL) was accurately measured to a column (1 cm in diameter) packed with 1.5 g neutral aluminum oxide (100—120 mesh), and

eluted with 60% methanol. The eluate was collected into 10 mL volumetric flask, collected about 9 mL, added with 1 drop phosphate acid and 60% methanol to scale, mixed, and filtered with 0.45 μm film. The successive filtrate was used as the sample solutions.

Preparation of standard solutions

The emetine hydrochloride and cephaeline hydrochloride reference substances were weighed accurately and dissolved in methanol, and then diluted quantitatively and stepwise with mobile phase to obtain a solution equivalent to 10 μg/mL emetine hydrochloride and 10 μg/mL cephaeline hydrochloride.

HPLC analysis

Chromatographic conditions The mobile phase was composed of methanol-acetonitrile-0.1% phosphate acid (9:3:88) in isocratic elution mode with flow rate of 1 mL/min. The effluent from the column was detected by a diode array detector and the detection wavelength was set at 205 nm. The temperature of column was kept at 40 ℃. Reference solutions and ipecac crude drug solution or ipecac extract solution (10 μL), ipecac liquid extract solution and Ipecac tincture (2 μL), were accurately injected into the column, respectively, and the chromatography was recorded.

Linearity The series of reference solutions comprising cephaeline hydrochloride and emetine hydrochloride reference solution were used to determine linear range of the analytes. The results were summarized in Table 1 and good correlations were found between the peak area (y) and concentration of tested compounds (x) (r = 0.999 97) with the test ranges. The limit of detection (LOD) and the lower limit of quantification (LLOQ) values of individual compounds (Table 1) clearly indicated that the analytical method was acceptable with excellent sensitivity.

Table 1 Linearity range, calibration equation, LLOQ, and LOD of cephaeline hydrochloride and emetine hydrochloride

Compounds	Linearity range / μg	Calibration equation	LLOQ / μg	LOD / μg
cephaeline hydrochloride	0.014 56—0.2184	y = 8 184 939.7 x + 1642	0.0060	0.0020
emetine hydrochloride	0.0321—0.321	y = 6 856 886.5 x − 18 354	0.0075	0.0025

Repeatability test Repeatability of this method was obtained by analyzing six different samples (crude drug, Batch No. 11052352) at 100% of the test concentration using the same preparation procedure. The average contents of cephaeline hydrochloride and emetine hydrochloride were 6.88 and 17.24 mg/g, and the RSD values were 0.82% and 1.05%, respectively, which satisfied the criteria of quantitative analysis.

Stability test The peak areas of two alkaloids in sample solutions were analyzed in 0, 10, 12, and 24 h. The RSD values of cephaeline hydrochloride and emetine hydrochloride were 0.32% and 0.51%, respectively. The results suggested that it was feasible to analyze samples within 24 h.

Accuracy test The accuracy of the method was validated by measuring recovery through standard addition method. Different amounts of the reference substances were spiked into nine samples (crude drug, Batch No. 11052352), and then the test solution was prepared. The extracted solution was analyzed by the proposed HPLC method. Quantity of each component was subsequently obtained using the corresponding calibration plots. The recoveries of cephaeline hydrochloride and emetine hydrochloride were 96.93% and 99.47%, and the RSD values were 1.31% and 2.02%, respectively (Tables 2 and 3). The above results exhibited the reliability and accuracy for the measurement of these constituents.

Methodology validation of ipecac preparations

Ipecac extract, ipecac liquid extract, and ipecac tincture are the ipecac preparations extracted with dilute ethanol solution and made into different preparation forms. Only according to the labeled contents, properly amount of samples was measured and diluted to the concentration similar to crude drug sample solutions. So the methodology validation of ipecac preparations is the same as that of ipecac crude drug.

Sample analysis

Samples of ipecac and its preparations from 11 batches were determined by HPLC and the contents were calculated with external standard method. As shown in Table 4, the contents of cephaeline and emetine, also the content ratio of cephaeline to emetine, varied significantly from different origins.

Table 2 Results of recovery for cephaeline hydrochloride in ipecac crude drug

Added amount / μg	Sample content / μg	Total content / μg	Recovery / %	Average recovery / %	RSD / %
181.4	337.1	513.1	97.02		
181.4	335.7	511.9	97.13		
181.4	339.2	515.5	97.19		
324.0	340.6	654.4	96.85		
324.0	333.7	645.9	96.36	96.93	1.31
324.0	319.9	629.1	95.43		
453.6	330.9	762.9	95.24		
453.6	344.7	787.6	97.64		
453.6	352.3	803.8	99.54		

Table 3 Results of recovery for emetine hydrochloride in ipecac crude drug

Added amount / μg	Sample content / μg	Total content / μg	Recovery / %	Average recovery / %	RSD / %
470.4	844.8	1306.6	98.17		
470.4	841.3	1315.9	100.89		
470.4	849.9	1309.6	97.73		
784.0	853.4	1621.1	97.92		
784.0	836.1	1621.1	100.13	99.47	2.02
784.0	801.7	1611.2	103.25		
1097.6	829.2	1941.4	101.33		
1097.6	863.7	1941.8	98.22		
1097.6	882.7	1954.3	97.63		

Table 4 Contents of two alkaloids in ipecac and its preparations

Samples	Cephaeline / %	Emetine / %	Sum / %	Cephaeline / Emetine
crude drug 1 (Costa Rica)	2.51 ± 0.02	1.17 ± 0.01	3.68 ± 0.02	2.15
crude drug 2 (Costa Rica)	3.20 ± 0.03	1.51 ± 0.01	4.71 ± 0.03	2.12
crude drug 3 (origin unknown)	2.33 ± 0.02	1.46 ± 0.01	3.79 ± 0.02	1.60
crude drug 4 (Brazil)	0.74 ± 0.01	1.47 ± 0.02	2.21 ± 0.02	0.50
crude drug 5 (Brazil)	0.70 ± 0.01	1.68 ± 0.02	2.38 ± 0.02	0.42
crude drug 6 (India)	1.72 ± 0.02	1.60 ± 0.02	3.32 ± 0.01	1.08
liquid extract (batch No. 20120001, crude drug origin unknown)	0.45 ± 0 .01	1.97 ± 0.02	2.42 ± 0.02	0.23
liquid extract (batch No. 20120002, crude drug Brazil)	0.84 ± 0.01	1.53 ± 0.01	2.37 ± 0.01	0.55
liquid extract (batch No. 20120003, crude drug Costa Rica)	1.40 ± 0.01	0.76 ± 0.01	2.16 ± 0.01	1.84
tincture (batch No. 20120004, crude drug origin unknown)	0.07 ± 0.01	0.04 ± 0.01	0.11 ± 0.01	1.75
extract (batch No. 20120005, crude drug origin unknown)	2.70 ± 0.03	1.48 ± 0.02	4.18 ± 0.03	1.82

Results and discussion

Comparison between HPLC and acid-base titration methods in ipecac crude drug

According to USP, the content of emetine and cephaeline together is not less than 90.0% of the total alkaloids. In JP, the total content of cephaeline and emetine is about 84% of the total alkaloids. We determined the content of total alkaloids and two alkaloids by different methods, the former using acid-base titration, and the latter using HPLC method, respectively (Table 5). According to reports, the results of total alkaloids should be higher than the sum of the two alkaloids by HPLC method, but the results were opposite. The problem will be discussed.

Table 5 Comparison of HPLC to titration method in ipecac crude drug

Samples	Methods	Alkaloids / %	Cephaeline / %	Emetine / %	Sum of two alkaloids / %	Ratio of HPLC to titration / %
Ipecac	Titration	2.98				124.83
(Costa Rica)	HPLC		2.54	1.18	3.72	
Ipecac	Titration	1.87				118.18
(Brazil)	HPLC		0.74	1.47	2.21	

Steps of acid-base titration method

The preparation of the test solution by acid-base titration method in *China Registered Standard* is, "To weigh accurately 7.5 g of finely powdered ipecac, add 100 mL of ether, shake the mixture for 5 min, then add 5 mL of 6 N ammonia hydroxide, shake it for 1 h; add 5 mL of water, shake violently, separated the ether layer, filter through cotton, collect the filtrate into flasks, wash the residue with ether two times, each 25 mL, combine ether extraction, dry ...".

Shortcomings in acid-base titration method for preparation of sample solutions

There are some shortcomings in the sample processing. First, the method using ether as extraction solvent with low penetrating force into plant cells could not directly extract alkaloids from plant powder. Second, the purpose of adding a small amount of ammonia test solution is to make alkaloids free in the plant cells and soluble in ether. According to the reasonable procedure, ammonia test solution should be added first, and then ether. But the operation is opposite. So the ether was coated by fine powder, and the ammonia test solution could not contact fully with fine powder, which brought about alkaloids extracted incompletely. Third, in addition to using the extraction solvent penetration, the yield of alkaloids from plant fine powder also depends on external forces, e.g. heat or ultrasonic. In *China Registered Standard*, neither rule the shake frequency nor shake strength, so that the results could vary from different individuals. Consequently, it is difficult to ensure the reproducibility. And fourth, when ipecac fine powder, ether, water, and ammonia test solution were mixed with each other, an emulsions mixture was produced. It is difficult to isolate ether extracts in the upper layer quantitatively, which affects the accuracy and reproducibility.

From the above, there are some uncertain factors affecting acid-base titration method. This may explain why the contents of total alkaloids obtained using acid-base titration is lower than those obtained using HPLC method.

Optimization of chromatographic conditions

Column types, mobile phase compositions, and column temperature were optimized respectively to achieve good separation within a shorter analysis time. Different types of column including Shimadzu ODS-VP column (150 mm × 4.6 mm, 5 μm), Dikma diamonsil C_{18} column (150 mm × 4.6 mm, 5 μm), and Shimadzu ODS-SP column (150 mm × 4.6 mm, 5 μm) were evaluated. Typical chromatograms are presented in Fig. 2. The both alkaloids were well separated and suitable to the retained time under established chromatographic conditions.

The UV spectrum of emetine and cephaeline by scanning with diode array detector showed a similar spectrum and had three absorption peaks: 202, (228 ± 3),

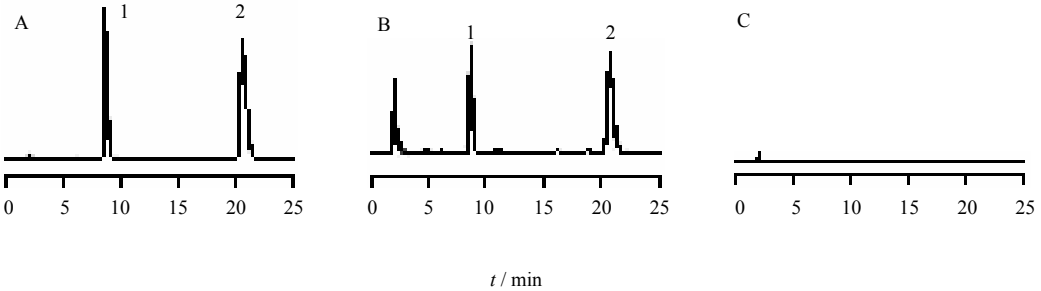

Fig. 2 HPLC chromatograms of alkaloids reference substance (A), ipecac crude herb (B), and negative sample (C)

1: cephaeline hydrochloride 2: emetine hydrochloride

and (282 ± 1) nm (Fig. 3). The detective sensitivity at 202 nm is six times for that at (228 ± 3) nm and 12 times for that at (282 ± 1) nm, respectively. In order to enhance the sensitivity, decrease the sample amount, and increase the column life expectancy, the most appropriate wavelength was set at 205 nm.

Determination of ultrasonic extraction time

In order to fully extract the sample, ultrasonic extraction time was investigated and confirmed at 30 min by comparing the contents in a series of different ultrasonic extraction time.

Role and amount of neutral alumina column

During the content determination of ipecac crude drug and its preparations by HPLC, a strong retention of unknown peak was found about 60 min in chromatogram, in which the area was about 30% of total peak

areas (Fig. 4A). To shorten the determination time, neutral alumina column was used to remove the unknown peak. The results showed that the contents of cephaeline hydrochloride and emetine hydrochloride were the same by comparing passed through alumina column with no passed one (Fig. 4B). The amount of alumina was 1.5 g, with a good effect to remove impurities.

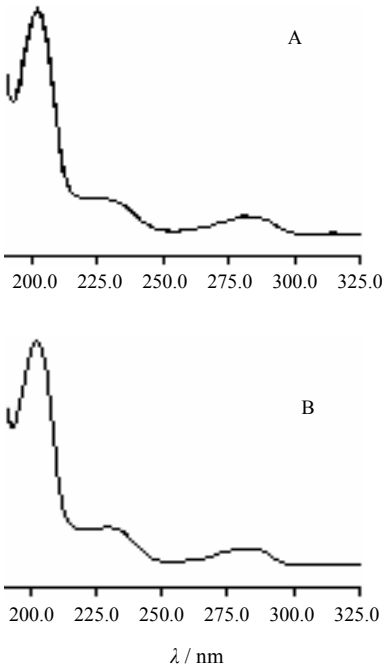

Fig. 3 UV spectra of cephaeline hydrochloride (A) and emetine hydrochloride (B)

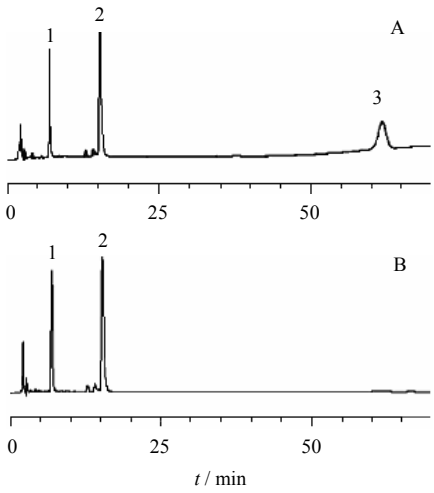

Fig. 4 HPLC chromatograms of ipecac crude drug with no passed (A) and passed (B) alumina column

1: cephaeline hydrochloride 2: emetine hydrochloride

3: unknown peak

Conclusion

In comparison with USP, EP, *China Registered Standard*, and previously published literatures, the research paper is characteristic and innovative. An RP-HPLC method was first developed to simultaneously determine the amount of cephaeline and emetine in ipecac and its preparations, using isocratic elution and mobile phase containing no buffer salt and no ion-pair solvent, so there is no damage to chromatographic column caused by buffer salt and ion-pair solvent. The detector was set at 205 nm, the detection sensitivity is 12 times more than that in previously published research literature at 285 nm, which greatly reduced the sample amount, improved the filtration speed, and reduced column-blocking compounds.

Using one-step ultrasonic extraction for 30 min and alumina column in the sample pretreatment protocol, the time was less than 1 h compared with USP (12—16 h), and the volume of organic solvents (60% methanol) was consumed 35 mL compared with USP (655 mL). So the sample preparation method is simpler, more efficient, more economical, and more environmental friendly impact. More importantly, this method could overcome the deficiencies of the legal standard and exactly determine the alkaloid content. The proposed method had been elucidated to be a simple, sensitive, accurate, and reliable quality control procedure for ipecac and ipecac preparations.

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