Chromatographic Separation of (−)-Ephedrine and (+)-Pseudoephedrine in the Traditional Chinese Medicinal Preparation Jiketing Granule

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(−)-Ephedrine (EP) and (+)-Pseudoephedrine (PEP) in Jiketing granule were separated by TLC, RP-HPLC, GC, HPLC-MS, and the chromatographic phenomenon was interpreted by the topological indices. The observed chromatographic separations of investigated components were compared. This comparison indicated that simply methods as C_{18} RP-HPLC and GC are the more effective techniques, and more suitable than others for this preparation, for the separation of two alkaloids.

Key words (−)-ephedrine (EP); (+)-pseudoephedrine (PEP); chromatographic separation; Jiketing granule

The prescription of traditional Chinese Medicine (TCM) can be defined as a preparation, which organically combines various crude drugs for the prevention and treatment of disease in accordance with a certain principle. Jiketing Granule is a famous preparation that contains 10 crude drugs, including herba ephedrae (Mahuang), semen armeniacae amarum (Kuxingren), radix stemonae (Baibu), *et al.* The clinical trial indicates that it is effectively used for antitussive. Ephedra herb is one of the main ingredients in the formulation, which has usually been used for antitussive, expectorant, antipyretic, analgesics, and bronchitis reagents. The main active components are benzylamine alkaloids in which (−)-ephedrine (EP) and (+)-pseudoephedrine (PEP) (Fig. 1) are the main bioactive components of the total alkaloids in the plant.[1)] Because the contents of EP and PEP are quite different in herba ephedrae from different area establishing quantitative standard of these two components for Kechuanting Granule is necessary, and would be a reference to producer to ensure product quality.

Several reports have been published on the quantitative determination of herba ephedrae alkaloids using TLC,[2,3)] HPLC,[4,5)] GC,[6)] Capillary Electrophoresis (CE).[7)] These methods are not suitable for separation of EP and PEP in this preparation because of the interference caused by other complicated chemical constituents. Therefore, in this paper, different chromatographic methods, including HPLC, GC, TLC, LC-MS had been investigated for separation of EP and PEP in Jiketing Granule. The results indicated that the HPLC and GC methods are specific and accurate as compared to TLC, LC-MS. Moreover, the sample pretreatment method proved to be suitable, which adopted to enhance sensitivity and to remove interference that influence on the detection of the target compounds. Further research on topological indices shows that HPLC and GC are highly desirable, suitable for quality control of Jiketing Granule.

Experimental

Material and Reagents If not otherwise specified, water is distilled. Methanol, acetonitrile were HPLC grade (Tedia Company, U.S.A.) and other chemicals were analysis grade. Jiketing Granule was developed by department of Pharmaceutics of China Pharmaceutical University. EP and PEP (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) were used as standards.

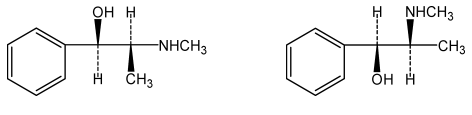

Fig. 1. Chemical Structures of EP and PEP

Instrumentation and Chromatographic Condition HPLC System: The HPLC system consisted of two delivery pumps (Shimadzu LC-10AD, Japan), a UV detector (Shimadzu, LC-10AVP, Japan), a model 7725i manual injector valve with a 20 μl sample loop. The signals from the detector were connected and analyzed with a computer equipped with a software of N-2000 system (Zhejiang University).

The samples were separated with an Hypersil C_{18} Column (250 mm× 4.6 mm, 5 μm) supplied by Yilite Company, Dalian, China. The mobile phase was composed of acetonitrile–0.02 mol/l dihydro-potassium phosphate solution containing 3% triethylamine with the pH of 3.0 (4 : 96, v/v). The flow rate of 1.0 ml/min was used. The detector wavelength was set at 210 nm and column temperature was maintained at 40 °C. The injection volume was 20 μl.

GC System: GC analysis was performed using gas chromatograph (Shimadzu GC-14B) equipped with a electron capture detector (ECD) system. Data were recorded and analyzed by N2000 series (Zhejiang University). An Alltech EC^{TM}-1 capillary column (30 m×0.32 mm, 0.25 μm) was utilized. Nitrogen was used as the carrier gas at a flow rate of 27 ml/min. The oven temperature was operated at 100 °C for 1 min, then heated at the rate of 20 °C/min until to 220 °C. And injector was set at 230 °C and the detector was set at 250 °C. An aliquot (2 μl) of sample was injected with a split injection of 26 : 1.

LC-MS System: A Waters 1575 series LC system equipped with a binary pump was connected to a Waters 743 autosampler. Chromatographic separation was carried out at 40 °C using a Hypersil ODS analytical column (250×4.6 mm 5 μm) supplied by Yilite Company, Dalian, China. The mobile phase consisted of acetonitrile–0.25% ammonium acetate solution (80 : 20) with the rate of 1.0 ml/min.

Detections were performed by a Waters 717 mass spectrometer. For MS acquisitions, the column effluent was split by a zero-dead volume "T" connector, with approximately the flow rate of 0.3 ml/min to the mass spectrometer. The mass spectrometry detector (MSD) was equipped with an electrospray ionization (ESI) source. The ionization mode was positive. The interface and MSD parameters were as follow: capillary cove, 15 V; dry gas, N_2 (9 l/min); nebulizer pressure, 35 psi (N_2); dry gas temperature, 350 °C; target ion of (−)-ephedrine and (+)-pseudoephedrine, *m*/*z* 366; target ion of berberine hydrochloride (internal standard), *m*/*z* 336. All data acquired were processed by Waters Chemstation (MassLynx V4.0).The injection volume was 40 μl.

TLC System: TLC was performed on 20×20 cm glass backed silica gel 60, 0.2 mm thick TLC plates (Qingdao haiyang chemical factory subsidiary factory, Qingdao, China). The alkaloids of the sample were determined by TLC-scanning (Shimadzu, CS-910, Japan) with double wavelength reflection sawtooth scanning: transmitting wavelength, 520 nm; reflecting wavelength, 700 nm.

Aliquots (3 μl) of the extracts of the test samples were applied to the origin of the TLC plate manually using microsyringe. The plates were developed in an saturated tank with chloroform–methanol–concentrated aqueous ammonia (40 : 8 : 1, v/v) for 12 cm. The plates were then air dried at room

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temperature, spraied with ninhydrin solution (20%) and heated at 105 °C until the spots were clear.

Sample Preparation HPLC and LC-MS: The finely powdered sample (about 1 g) was transferred into a flask and dissolved with 10 ml water in an ultrasonic bathe for 5 min at 30 °C, added 100 ml mixture solution of 4 g sodium chloride and 10 g sodium hydroxide, and distilled. About 100 ml distilled solution was collected in flask contained 50ml 1% HCl concentrated to 20 ml. When UV detector was selected, the pH value of the solution was adjusted to 4 with 10% sodium hydroxide and concentrated to 5 ml. Then the solution was transferred into 10 ml volumetric flask and diluted with water as HPLC sample. Due to the requirements of MSD, 10% aqueous ammonia was added to adjust the pH value to 4 and concentrated to 5 ml. After the solution was transferred into 10 ml volumetric flask and diluted with water, 1 ml was accurately extracted and diluted into 100 ml as HPLC-MS sample. Finally, the two samples were filtered through a 0.45 μm filter membrane before analysis.

GC: The finely powdered sample (about 0.05 g) was transferred into a test tube and dissolved with 0.5 ml of water. After adding 100 μl of 2 mol/l sodium chloride solution and 100 μl of saturated sodium hydroxide solution and 3 ml organic mixture of *n*-Hexane and ethyl acetate (3 : 1), the solution was vortexed for 3 min and centrifuged at 3500 rpm for 5 min. After the organic layer was transferred, 3 ml organic mixture was again added into the solution and extracting process was repeated. All the organic layers were colleted and 50 μl of 6 mol/l hydrochloric acid was added. Then the mixture was evaporated to dryness in a water bath at 60 °C under a stream of nitrogen. The residue was added 200 μl ethyl acetate and 100 μl trifluoroaceticanhydride, then reacted for 30 min in a water bath at 50 °C. After evaporated to dryness under the stream of nitrogen, the residue was reconstituted in 1 ml of ethyl acetate and centrifuged (12000 rpm) for 5 min before analysis.

TLC: The finely powdered sample (about 2 g) was a dissolved with 5 ml mixture solution (5 ml water solution containing 1 ml ammonia solution and 0.2 g sodium chloride). Then the solution was added 6 ml ether and shaken. After the organic layer was transferred, the water layer was again added 6 ml ether. According the above, the water layer was extracted with 6 ml ether three times. All of the extract layers were collected together, and added 3 ml 3% hydrochloric acid solution. After shaken and placed for 30 min, the water layer was extracted and dried. The residue was dissolved with methanol, transferred into 5 ml volumetric flask and diluted with methanol.

Reference Tests In order to check in the interference from other drugs and excipients used in the formulation, other medical materials except herba ephedra were weighted and prepared according to the requires of different chromatographic methods to the negative control samples. The results showed that there was no interference of the negative control samples under chromatographic condition of HPLC, GC, LC-MS, TLC.

Results and Discussion

Selection of EP and PEP for Quality Control EP and PEP were selected for quality control based on two reasons: 1) it had been reported that they have the highest biological activity in antitussive and the clinical trials showed that the existence of EP and PEP closely related to pharmacology effects of Jiketing Granule. 2) The contents of EP and PEP were quite different because a number of species comprise the source of herba ephedrae on the market and the diverse geographical origins. For this reason, establishing regulate quantitative standard of these two components is necessary for selecting the GAP foundation and ensure product quality.

Evaluation of Chromatographic Condition HPLC: When acetonitrile and pure water were used as the mobile phase for HPLC, the peaks of PEP and EP were not separated. Therefore, the composition of mobile phase was optimized using different proportions of acetonitrile–dihydrogen potassium phosphate solution. Four percents of acetonitrle and 0.02 mol/l dihydrogen potassium phosphate solution were selected as an optimum ratio in the mobile phase to achieve good separation of the peaks and shorten the analytical time.

GC: When non-polar column (DB-5, 30 m×0.2 mm, 0.25 μm) or strong-polar column (DB-FFAP, 30 m×0.2 mm, 0.25 μm) was selected, the change of column temperature could not achieve good separation between PEP and EP. However, after reacted with trifluoroacetic anhydride, the peaks of PEP and EP were completely separated on non-polar column. And the pretreatment could augment signals of ECD to achieved high sensitivity. So, we selected the method of derivatization to analysis PEP and EP in Jiketing Granule.

Comparison of the Methods HPLC: Under the developed HPLC conditions, PEP and EP had good separation. PEP and EP calibration curves both showed good linear regressions. Furthermore, the results demonstrated that HPLC analytical method is reproducible with good accuracy. In the pretreatment of samples, in terms of physicochemical properties of PEP and EP which are volatile, distillation and acidification were adopted to remove most of the polar interferences and to avoid a loss of sample.

GC: Owing to the low boiling points of PEP and EP, the developed GC method was applied to the simultaneous determination of the two ingredients in Jiketing Granule. The method of derivatization was selected to achieve good separation and high sensitivity.

HPLC-MS: Due to the same *m*/*z* of the both and the requirements of HPLC-MS to mobile phase, PEP and EP were hardly separated on reversed-phase column. But the determination total of PEP and EP by HPLC-MS was consistent to that by HPLC and GC. Recently our research team was conducting further investigations on alkaline column to have good separate between PEP and EP.

TLC: This method was not suitable to determination of PEP and EP in Jiketing Granule due to their similar polarity. And TLC plates must be spraied with ninhydrin solution (20%) and heated at 105 °C until the spots were clear before being scanned, so TLC had poor reproducibility.

Validation of the Methods We validated the methods from linearity, limit of detection, limit of quantitative, recovery, within-day and between-day accuracy, and summarized the results of studies in Tables 1 and 2. The stability of the solution was determination at 0, 4, 6 and 8 h. And the relative standard deviations (RSD) of peaks area were up to prescription. The result showed that the sample solution was stable for 8 h (at 5 °C).

The results of determination of Jiketing granule were showed in Table 3 and all the chromatograms of three methods were showed in Fig. 2.

Calculation of Topological Indices The topological indices, which descend from a graph theory, are the simplest way of a structural description of a molecule. Due to encode the structural information of a molecule, the topological indices characterize a molecule by a simple number. And the topological indices were connected with the properties of a molecule, so the numerical values of the topological indices can be used to predict the chromatographic separation of the investigated sample.

The topological indices based on connectivity and distance matrix: A, W, $^{h}x^{v}$, *etc.* Especially, $^{h}x^{v}$ was extensively used to predict chromatographic retention factors by the induce of the experiential formula concerning $^{h}x^{v}$.[8]

The topological indexes shows that PEP and EP possess the same numerical values of topological indices $^{h}x^{v}$, because PEP and EP are isomeric structures. To HPLC, when the mo-

Table 1. The Results of Validation of Four Methods

Method of chromatography	Limit of detection (ng)		Limit of quantitative (ng)		Recovery (%)		Within-day accuracy (%)		Between-day accuracy (%)	
	EP	PEP	EP	PEP	EP	PEP	EP	PEP	EP	PEP
HPLC	0.3	0.4	0.5	0.6	98.9	99.6	1.2	0.9	1.7	1.9
GC	0.03	0.04	0.05	0.06	99.7	101	0.6	0.7	0.8	1.0
TLC	83.4		166.7		98.2		6.2		7.4	
LC-MS	0.3		0.5		98.7		6.7		10.2	

Table 2. Linear Regression Equation and Linear Range of Four Methods

Method of chromatography	Linear regression equation		Linear range (μg/ml)	
	EP	PEP	EP	PEP
HPLC	$A^{a)}=16898c-4271.4$, $r=0.9999$ ($n=5$)	$A=17322c-5023.3$, $r=0.9998$ ($n=5$)	30.0—70.0	10.0—50.0
GC	$A=86494c-9783.4$, $r=0.9999$ ($n=5$)	$A=89953c-1349.4$, $r=0.9998$ ($n=5$)	1.0—5.0	0.5—4.0
TLC	$A=177517c-166614$		166.7—833.3	
LC-MS	$F^{b)}=0.0007c+0.0619$		0.5—2.0	

a) Area of EP (or PEP), *b*) Peak area ratio of EP (and PEP) to IS, EP: (−)-ephedrine, PEP: (+)-pseudoephedrine.

Table 3. The Content of EP and PEP in Jiketing Granule by Four Methods

Method of chromatography	Content (μg/g)		RSD (%)	
	EP	PEP	EP	PEP
HPLC	106.1	266.1	1.8	0.5
GC	107.5	267.7	0.8	0.3
TLC	368.9		4.2	
LC-MS	369.7		1.9	

bile phase was only composed of pure water and acetonitrile, ${}^{h}x^{v}$ has good connection with retention factors.[8] Due to the same values of ${}^{h}x^{v}$ of PEP and EP, PEP and EP had poor separation on C_{18} column. However, adding dihydro-potassium phosphate into mobile phase maybe change the connection between ${}^{h}x^{v}$ and chromatographic retention factors, so PEP and EP were separated. The same thing possibly happened to TLC and GC. In TLC chromatographic graph, only one spot was observed, which perhaps resulted from the same values of ${}^{h}x^{v}$, and *Rm* of TLC could be induced from these values. To gas chromatography, when non-polar column was selected, the peaks of PEP and EP were not completely separated because retention factors could be induced from the same ${}^{h}x^{v}$. After PEP and EP were reacted with trifluoroaceticanhydride, N–H in their structures was changed to $\mathrm{N{-}\overset{\overset{O}{\|}}{C}{-}F_3}$. The structural changes could achieve the good shapes of chromatographic peaks and make these compounds completely separated. So the topological indices could be used to predict elementarily the chromatographic retention factors of compounds.

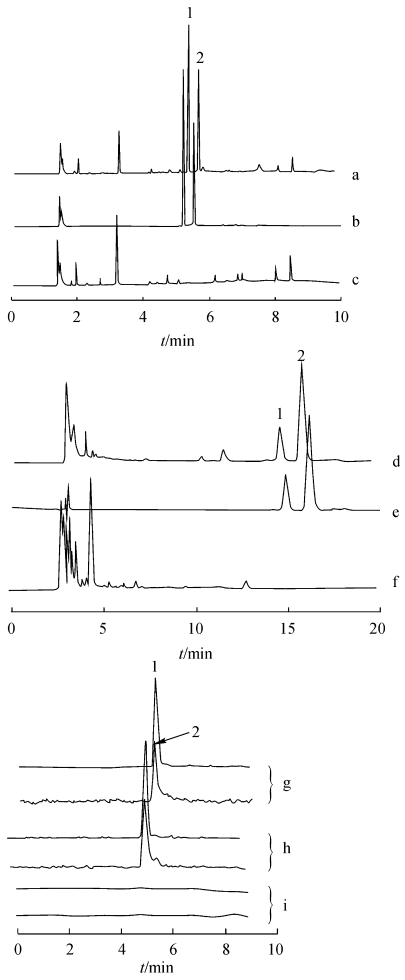

Fig. 2. Chromatograms of EP and PEP from Jiketing Granule

a, sample; b, standards; c, ephedra herb deficient sample; above all of GC chromatograms. d, sample; e, standards; f, ephedra herb deficient sample; above all of HPLC chromatograms. g, sample; h, standards; i, ephedra herb deficient sample; above all of HPLC-MS chromatograms. 1, EP; 2, PEP.

Conclusion

The chromatographic conditions which were applied in the HPLC and GC could be used to separate PEP and EP in

Jiketing Granule. Additionally, the structures of the particular of PEP and EP were described by topological index ${}^{h}x^{v}$ which were applied to interpret the chromatographic phenomenon.

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