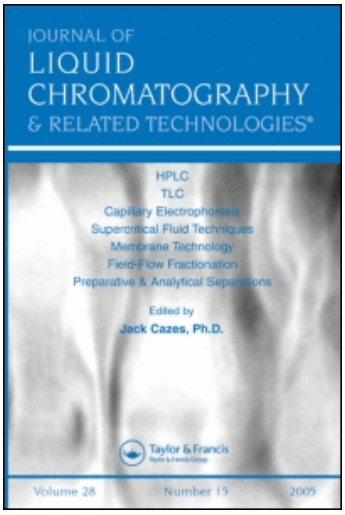

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SIMULTANEOUS SEPARATION AND DETERMINATION OF EPHEDRINE ALKALOIDS AND TETRAMETHYL-PYRAZINE IN *EPHEDRA SINICA* STAPF BY HPLC

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ABSTRACT

A simple, sensitive, and reliable high performance liquid chromatography (HPLC) method was first developed for simultaneous determination of Ephedrine alkaloids and 2,3,5,6-tetramethylpyrazine (TMP) in *Ephedra sinica* Stapf. The HPLC separation was performed on a reversed phase C18 column (Nova-Pak® C18, 150 mm × 3.9 mm I.D.) with a duplicate gradient composed of methanol and phosphate buffer solution (PBS, 0.03 mol/L KH_2PO_4-acetic acid-triethyl amine = 400:0.25:0.1, v:v:v, pH = 6.0) to elute the solutes with a flow rate of 0.8 mL/min. The detection wavelength was set at 210 nm.

Regression equations revealed linear relationships (correlation coefficients: 0.991 ~ 0.998) between the peak area of each

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constituent (E, PE, NE, NPE, TMP) and its concentration. The detection limits for E, PE, NE, NPE, and TMP were 0.4, 0.1, 0.03, 0.02, 0.03 μg/mL, respectively at a signal-to-noise ratio of 3:1, and the recoveries ranged between 96.0–104.5%. The contents of E, PE, NE, NPE, and TMP in *Ephedra sinica* Stapf extracts were measured 0.480 g/100 g (2.5%, RSD), 0.090 g/100 g (3.0%, RSD), 0.090 g/100 g (2.4%, RSD), 0.060 g/100 g (2.2%, RSD), and 0.022 g/100 g (1.5%, RSD), respectively.

INTRODUCTION

Ephedrae herba (*Ma-Huang*) is a traditional Chinese medicine derived from the aerial parts of *Ephedra sinica* Stapf, *E. equisetina* Bunge, *E. intermedia* var. *tibetica* Stapf and *E. distachya* L. It has been used medicinally as a diaphoretic, stimulant, and antiashmatic. The medicinal properties of this Ephedra species are due to their contents of Ephedrine alkaloids, such as ephedrine (E), pseudoephedrine (PE), methylephedrine (ME), norephedrine (NE), and norpseudoephedrine (NPE) (1). Several methods have been reported for the determination of Ephedrine alkaloids, such as nonchromatographic (colorimetric, titrimetric, and gravimetric) methods (2–4), thin-layer chromatography (5), high-performance liquid chromatography (6–10), gas chromatography (11–13), capillary electrophoresis (14), and nuclear magnetic resonance spectrometry (NMR) (15). Most of these methods seem to be unsuitable for the quantitative determination of these stereoisomers, owing to either poor resolution or low sensitivity. Furthermore, the known methods require tedious pretreatment of Ephedra extracts before analysis, leading to a possible source of error.

Another important alkaloid, 2,3,5,6-tetramethylpyrazine (TMP), has also been found in *Ephedrae herba* and plays a very important role in relieving ashma (16). Some articles analyzed TMP in *Ephedrae herba* as a kind of volatile oil (16–18), which was not an accurate and simple method for TMP quantification. In a previous paper, we have identified and quantified TMP in *Ephedra sinica* Stapf by HPLC and GC-MS (19), but TMP in *Ephedra sinica* Stapf has never been simultaneously determined with Ephedrine alkaloids. In the present paper, TMP was simultaneously separated and identified with Ephedrine alkaloids by reversed high performance liquid chromatography. Our method differs from those reported earlier with regard to column type, mobile phase composition, column temperature and application.

To our knowledge, this is the first study of its kind to quantify the four principle Ephedrine alkaloids and TMP in *Ephedra sinica* Stapf using HPLC, which appears to be a suitable method for the analysis of Chinese herbal preparations, especially for large numbers of samples and for quality control in pharmaceutical plants.

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EXPERIMENTAL

Materials

Norephedrine hydrochloride, Norpseudoephedrine hydrochloride, Ephedrine hydrochloride, Pseudoephedrine hydrochloride, and TMP were purchased from Sigma. Each alkaloid showed a single peak on HPLC chromatograms. Methanol was of HPLC grade. Other chemicals were of analytical grade. *Ephedra sinica* Stapf was purchased from Si-chuan province of China.

Preparation of *Ephedra sinica* Stapf Extracts

2.4 g raw medicinal material of *Ephedra sinica* Stapf was crushed into small pieces and then extracted with 100 mL alkaline water-chloroform (1 : 1) by refluxing on a water bath at 80°C for 1.0 hr, then placed in an ultrasonic bath for 10 min. Extraction was repeated two times. The total organic solvent was combined and removed with a rotary evaporator at 40°C under vacuum. The residue was dissolved with methanol and diluted to appropriate concentration and filtered through a 0.45 μm filter membrane before HPLC analysis.

High Performance Liquid Chromatography (HPLC)

The mobile phase was delivered by a quaternary gradient pump (HP 1100 Chromatograph) at a flow rate of 0.8 mL/min and was used together with a diode array detector at wavelength of 210 nm. The sample was introduced by a 20 μL injection valve into the column (Zorbax SB-C18, 4.6 mm i.d. × 250 mm, 5 μm particles), which was placed in a column oven. The column temperature was set at 30°C. Data acquisition was performed by a commercial interface (HP Chemstation data analysis system). The mobile phase was filtered by a Millipore vacuum filter system equipped with a 0.45 μm filter before use.

RESULTS AND DISCUSSION

Optimization of Separation of Ephedrine Alkaloids and TMP

According to the reported literature, ephedrine, and pseudoephedrine can not be separated using methanol-water as mobile phase on C18 column. In the present paper, we used phosphate, acetic acid, and triethyl amine to optimize the separation conditions. Triethyl amine, which can decrease the interaction between

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the hydroxy group of ephedrine alkaloid with the octadecylsilica stationary phase, played an important role in the separation of ephedrine alkaloids. The polarity of ephedrine alkaloids and TMP was very different, so we used a different organic solvent composition to elute these solutes. In the present paper, a duplicate gradient composed of methanol and phosphate buffer solution (PBS, 0.03 mol/L KH_2PO_4-acetic acid-triethyl amine = 400 : 0.25 : 0.1, v : v : v, pH = 6.0) was used as mobile phase to separate ephedrine alkaloids and TMP in *Ephedra sinica* Stapf. The elution program was shown in Table 1. In this program, the mobile phase composed of 15% methanol was first used to resolve the impurities and then the methanol composition was increased to 25% to resolve the ephedrine alkaloids. Finally, the methanol composition was increased to 45% to speed up the elution of TMP. Under this duplicate gradient, four ephedrine alkaloids and TMP were well separated within a separation time of 32 min.

The UV spectra of ephedrine alkaloids have maximum absorption at 256 nm and 210 nm. TMP has maximum absorption at 215 nm and 290 nm. In the present paper, we selected 210 nm for detection.

Identification of E, PE, NE, NPE, and TMP in *Ephedra sinica* Stapf

The chloroform extracts of *Ephedra sinica* Stapf dissolved in methanol were injected into HPLC. The chromatogram was shown in Fig. 1. Comparing the retention time of the extracts and the standards (Fig. 2), peak 1, 2, 3, 4, and 5 were corresponding to NE, NPE, E, PE, and TMP, respectively. Further identification was confirmed by comparing the UV spectrum of extracts with that of authentic compounds. It was found that the UV spectra of peak 1, 2, 3, 4, and 5 in Fig. 1 were very similar to those of standards NE, NPE, E, PE, and TMP, respectively.

By comparing with the retention time and the UV spectra of the standards, the peaks with retention time at 12.27 min, 13.77 min, 15.64 min, 17.52 min, and 31.29 min were identified as NE, NPE, E, PE, and TMP, respectively. Because we

Table 1. The Elution Program of the Mobile Phase

Methanol (%)	PBS (%)	Time (min)	Flow Rate (mL/min)
15	85	0	0.8
15	85	12	0.8
25	75	15	0.8
25	75	19	0.8
45	55	19.1	0.8

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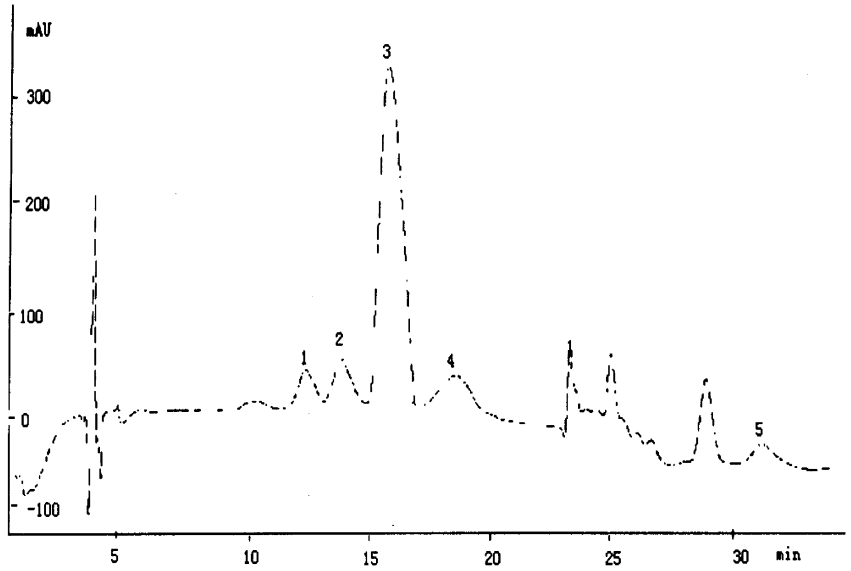

Figure 1. Chromatogram of the *Ephedra sinica* Stapf extracts. Column: (Zorbax Rx-Sil, 250 mm × 4.6 mm I.D., 5 μm); Mobile phase: a duplicate gradient of methanol and phosphate buffer solution (PBS, 0.03 mol/L KH_2PO_4-acetic acid-triethyl amine = 400 : 0.25 : 0.1, v : v : v, pH = 6.0); Flow rate: 0.8 mL/min; Detector: DAD, 210 nm. Peak: 1-NE, 2-NPE, 3-E, 4-PE, 5-TMP.

used a duplicate gradient composed of methanol and PBS as mobile phase, and we used short wavelength (210 nm) for detection, the baseline was shifted a little. The peak with retention time at 23.5 min in the chromatograms was caused by the solvent. However, these did not affect the determination of the interested compounds.

The Quantitative Analysis of E, PE, NE, NPE, and TMP in *Ephedra sinica* Stapf

The contents of NE, NPE, E, PE, and TMP in *Ephedra sinica* Stapf were quantified from the corresponding peak area, using linear equations. For this purpose, standard solutions of pure samples of NE, NPE, E, PE, and TMP, in variable concentrations, were run three times, respectively. The average peak area was calculated and plotted, and linear regression analysis was performed. The linear relationship between the concentrations of these five compounds and the

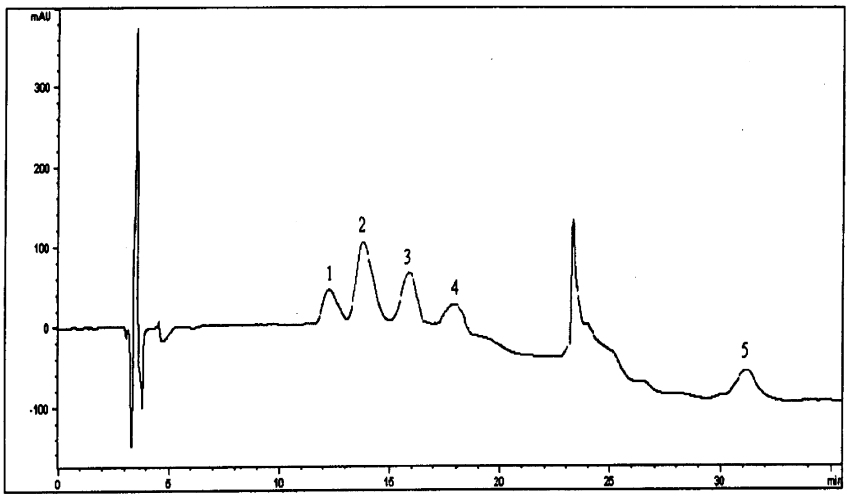

Figure 2. Chromatogram of the ephedrine alkaloids standards. Peak: 1-NE, 2-NPE, 3-E, 4-PE, 5-TMP. Chromatographic conditions were the same as Fig. 1.

corresponding peak areas were found in the concentration range of 6–200 μg/mL for ephedrine alkaloids, 2.6–264 μg/mL for TMP. The regression equations were as follows: $y = 99.8\ x - 135\ 6.6$ ($r = 0.9919$) for NE, $y = 81.5\ x - 544.7$ ($r = 0.9928$) for NPE, $y = 40.4\ x + 301.5$ ($r = 0.9982$) for E, $y = 43.1\ x + 568.3$ ($r = 0.9983$) for PE, $y = 7.6\ x + 19.1$ ($r = 0.9987$) for TMP, where y is the peak area for each compound and x is the concentration (μg/mL) of each compound.

Chromatographic precision, expressed as relative standard deviation (RSD), was calculated by injection of five replicates of the central point of the calibration curve. The detection limit was calculated by the formula: 3S.D/b, where S.D is the standard deviation, calculated by injecting 5 replicates of the lowest concentration solution of the calibration curve, and b is the value of the calibration curve slope. This formula is one of the possible algorithms for calculating the detection limit defined as the analyte amount that gives a signal-to-noise ratio = 3.

The detection limits for NE, NPE, E, PE, and TMP were 0.03, 0.02, 0.4, 0.1, and 0.03 μg/mL, respectively. This is, in principle, sufficient for analyte recognition.

The sample was filtered through a 0.45 μm membrane filter and then injected into the HPLC. The calculated contents of NE, NPE, E, PE, and TMP in *Ephedra sinica* Stapf were 0.090 g/100 g, 0.060 g/100 g, 0.480 g/100 g, 0.090 g/100 g, 0.022 g/100 g, respectively. Suitable amounts of standard NE,

NPE, E, PE, and TMP were added to the sample of known compound content and the whole was analyzed by the procedure stated above. Recovery was expressed, for each component, as the mean percentage ratio between the measured amounts and the actual ones. The average recoveries of NE, NPE, E, PE, and TMP were 96.0%, 102.2%, 96.2%, 102.4%, and 104.5%, respectively.

The reproducibility of the total procedure was tested using the sample of *Ephedra sinica* Stapf extracts. The relative standard deviations (RSD) were 2.4, 2.2, 2.5, 3.0, and 1.5% ($n = 5$) for NE, NPE, E, PE, and TMP, respectively. The results showed that the method had good recoveries and sensibility and could be readily utilized as a quality control method for *Ephedra sinica* Stapf.

CONCLUSION

Ephedrine alkaloids and tetramethylpyrazine in *Ephedra sinica* Stapf were simultaneously determined by HPLC without tedious pretreatment, such as derivatization. It was a simple, reliable, and quick method for determination of Ephedrine alkaloids and tetramethylpyrazine in ephedrine-containing medicinal herbs.

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