Separation and Determination of Ephedrine Alkaloids and Tetramethylpyrazine in *Ephedra sinica* Stapf by Gas Chromatography–Mass Spectrometry

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Abstract

A simple, sensitive, and reliable method using gas chromatography (GC)–mass spectrometry (MS) is developed for the simultaneous determination of ephedrine alkaloids and 2,3,5,6-tetramethylpyrazine (TMP) in *Ephedra sinica* Stapf. The sample is extracted with ethyl ether and submitted to GC–MS for identification and quantitation without derivatization. The column used for GC is an HP-5 (30.0 m × 250 µm × 0.25 µm, 5% phenyl methyl siloxane), and the carrier gas is helium. The detection limits for ephedrine, pseudoephedrine, and TMP are 0.4 ng, 0.7 ng, and 0.02 ng (signal-to-noise ratio of 3), respectively. The reproducibility of the total procedure is proved to be acceptable (RSD < 2%), and the recoveries are above 93%.

Introduction

Ma-Huang (*Ephedra herba*) 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 antiasthmatic. The medicinal properties of this Ephedra species are a result of their contents of ephedrine alkaloids (three pairs of optically active stereoisomers): (–)-ephedrine (E), (+)-pseudoephedrine (PE), (–)-norephedrine (NE), (+)-norpseudoephedrine (NPE), (+)-*N*-methylpseudoephedrine (MPE), and (–)-*N*-methylephedrine (ME) (1,2). A number of methods for the quantitative analysis of these ephedrine alkaloids have been reported, such as nonchromatographic methods (colorimetric, titrimetric, and gravimetric) (3–5), thin-layer chromatography (6,7), capillary electrophoresis (8–10), nuclear magnetic resonance spectrometry (11), high-performance liquid chromatography (HPLC), and gas chromatography (GC). An HPLC method (12–16) can give a baseline resolution of the alkaloids with the advantage of simple extraction and direct analysis of the alkaloids without derivatization. The reversed-phase HPLC (17–20) and ion-pair HPLC method (21,22) have been used for the determination of the alkaloids. The derivatization of alkaloids enhances the sensitivity and allows for the chiral HPLC separation of enantiomeric mixtures (23–26). However, the identification of the alkaloids is difficult to perform if there are no ephedrine standards.

GC methods for determining underivatized (27–28) and derivatized (29–30) ephedrine-type alkaloids in biological fluids have been published. J.M. Betz (31) et al. determined the ephedrine-type alkaloid content in dietary supplements containing *Ma-Huang* using a chiral GC method. The drawback, however, was that the elution times were greater than 60 min. GC methods for the chiral separation of enantiomers have also appeared recently (32,33). Methods using derivatized alkaloids allow for a much better separation of diastereomers, but these methods require a tedious pretreatment of extracts before analysis, which leads to a possible source of error.

Another important alkaloid, 2,3,5,6-tetramethylpyrazine (TMP), which was reported as the main active ingredient of another important traditional Chinese medicine (*Chuanxiong*), has also been found in *Ma-Huang* and plays a very important role in relieving asthma (34). Some articles analyzed TMP in *Ma-Huang* as a kind of volatile oil (34–36), which was not an accurate and simple method for TMP quantitation. In a previous study, we have identified and quantitated TMP in *Ephedra sinica* Stapf by HPLC and GC–mass spectrometry (MS) (37), but TMP in *Ma-Huang* has never been simultaneously determined with ephedrine alkaloids. In this study, a quick and sensitive GC–MS method for the simultaneous separation and determination of ephedrine alkaloids and TMP in *Ephedra sinica* Stapf was established without derivatization. Both the full-scan acquisition mode and selected ion monitoring (SIM) mode were used to identify and quantitate the ephedrine alkaloids and TMP in *Ephedra sinica* Stapf. To our knowledge, this is the first study of its kind to determine the principle ephedrine alkaloids and TMP in *Ephedra sinica* Stapf using GC–MS, which appears to be a suitable method for the analysis of Chinese herbal preparations, especially for large numbers of samples and 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. All alkaloids were purified by alkalization and recrystallization prior to use. Each alkaloid showed a single peak on GC chromatograms. All other chemicals employed were of analytical reagent grade.

Ephedra sinica Stapf was purchased from the Si-chuan province of China and was identified by Professor De-lin Liu (China Academy of Traditional Medicine, Beijing, China).

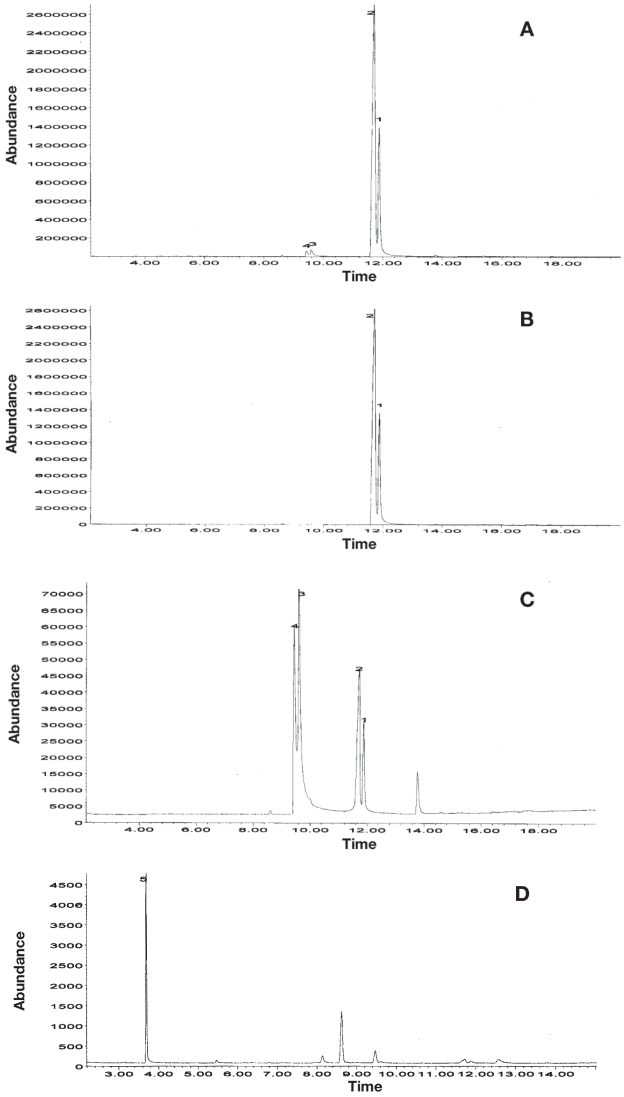

Figure 1. Chromatogram of *Ephedra herba* extracts: (A) TIC and extract ion chromatograms for *m/z* equal to (B) 58, (C) 44, and (D) 136.

Preparation of *Ephedra sinica* Stapf extracts

The extraction of ephedrine alkaloids from the plant was accomplished using a modified version of the method of Betz et al. (31). Two grams of raw medicinal material of *Ephedra sinica* Stapf was crushed into small pieces and then concentrated NH_4OH (5 mL) was added. The mixture was extracted with 60 mL ethanol–ethyl ether (1:2, v/v) by refluxing on a water bath for 30 min and was 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, then the residue was dissolved in 10 mL methanol. The methanol solution was filtered through a 0.45-µm membrane filter for GC–MS analysis.

GC–MS

The GC–mass selective detector (MSD) system consisted of a GC (HP 6890), an MSD (HP 5973), and an HP ChemStation data analysis. The running conditions were as follows.

Separation was performed on an HP-5 MS capillary column (30.0 m × 250 µm × 0.25 µm, 5% phenyl methyl siloxane) using the following temperature program. The program began at 60°C and then was raised to 100°C at 25°C/min. It was then raised to 110°C at 0.5°C/min and finally to 120°C at 1.0°C/min.

The injector temperature was 280°C, the MS source temperature 250°C, and the electron-impact ionization energy 70 eV. The column flow rate of carrier gas (He) was 1.0 mL/min with a split ratio of 30:1. Both the full-scan acquisition mode (*m/z* 35–400) and SIM mode were used in this study. Identification experiments were performed under full-scan acquisition mode (*m/z* 35–400) and quantitative analysis was performed under SIM mode. Ions at *m/z* 58, 44, and 136 were used as selected ions. Samples of 1 µL were injected manually.

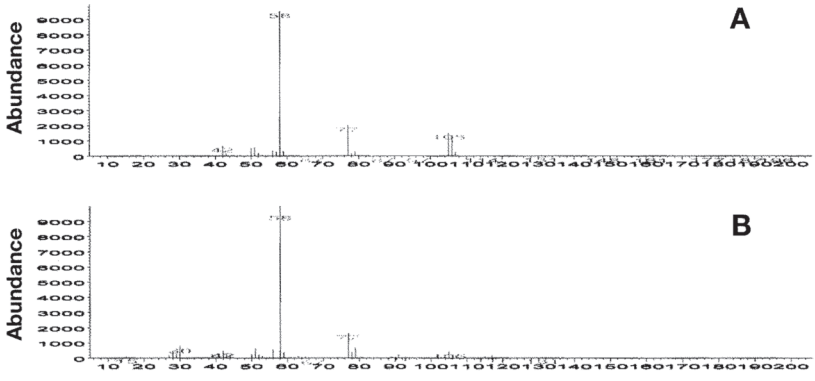

Figure 2. Mass spectrum of E in (A) *Ephedra herba* extract and (B) that of the authentic.

Results and Discussion

Identification of E, PE, NE, NPE, and TMP

The *Ephedra sinica* Stapf extracts dissolved in methanol were injected into the GC–MS and analyzed under full-scan acquisition mode. The total ion chromatogram (TIC) is shown in Figure 1A. The sensitivity and selectivity was improved greatly by using ion profiles extracted from the TIC. For example, when using an ion at *m/z* 44 to analyze Figure 1A, the sensitivity of peaks 3 and 4 were improved greatly. When using the extract ion at *m/z* 136 to analyze, peak 5 (which did not

appear in the TIC because of low content) was present in the chromatogram with high intensity (Figure 1D). When using the ion at *m/z* 58 to analyze, only peaks 1 and 2 were detected and well-separated (as shown in Figure 1B). Compared with the retention time of the standards, peaks 1, 2, 3, 4, and 5 corresponded with PE, E, NE, NPE, and TMP, respectively.

Further identification was confirmed by comparison of the mass spectra of the extracts with those of the authentic compounds. In the mass spectra (Figures 2–4), Figure A was the mass spectrum of the extracts and Figure B was the mass spectrum of the authentic compound. The mass spectrum patterns of peaks 2, 3, and 5 were very similar to those of E, NE, and TMP, respectively. Also, the mass spectrum patterns of peak 1 and 4 were similar to that of PE and NPE, respectively (figure omitted).

As showed in the mass spectra, E, PE, NE, and NPE exhibited $[M\text{-}C_6H_5CHOH\ ]^{+\cdot}$ as the base peak and TMP generated $[M]^{+\cdot}$ as the base peak, respectively. Other strong peaks of E and PE showed at *m/z* 77, 105, 117, and 42.

When using the SIM mode to analyze the extracts, ions at *m/z* 58, 44, and 136 were used as the selected ions. Approximately two orders of magnitude of more sensitivity for analysis was yielded than the full-scan approach previously described (figure was similar to Figure 1A). Therefore, the quantitative analysis was performed by GC–MS in the SIM mode.

Quantitation of E, PE, and TMP in *Ephedra sinica* Stapf

Detection was carried out in the electron-impact and SIM mode. Ions at *m/z* 58, 44, and 136 were used as selected ions. The contents of E, PE, and TMP in *Ephedra sinica* Stapf were quantitated from the corresponding peak area using linear equations. For this purpose, standard solutions of pure samples of 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 relationships between the concentration of these three compounds and the corresponding peak areas were found in the concentration range of 15.0 to 1520 µg/mL for E and PE and 0.3 to 13.2 µg/mL for TMP. The regression equations were as follows:

$$y = (1.46 \times 10^7)x - (2.80 \times 10^6)\ (r = 0.9985)\ \text{for E} \qquad \text{Eq. 1}$$

$$y = (1.76 \times 10^7)x - (3.31 \times 10^6)\ (r = 0.9970)\ \text{for PE} \qquad \text{Eq. 2}$$

$$y = (4.41 \times 10^4)x - (2.14 \times 10^4)\ (r = 0.9992)\ \text{for TMP} \qquad \text{Eq. 3}$$

where y is the peak area for each compound and x is the concentration of each compound (mg/mL for E and PE, µg/mL for TMP). The detection limit was calculated by the formula:

$$3SD/b \qquad \text{Eq. 4}$$

where SD is the standard deviation calculated by injecting five 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 of 3. The detection limits of E, PE, and TMP were 0.4, 0.7, and 0.02 ng, respectively, under SIM mode.

The sample was filtered through a 0.45-µm membrane filter and then injected into GC–MS, which was performed under SIM mode in order for detection. The calculated contents of E, PE, and TMP in *Ephedra sinica* Stapf were 0.65 g/100 g, 0.41 g/100 g, and 0.03 g/100 g, respectively. Suitable amounts of standard E, PE, and TMP were added to the sample of known compound content and the whole was analyzed by the procedure stated previously. Recovery was expressed for each component as the mean percentage ratio between the measured amounts and the actual ones. The average recoveries of E, PE, and TMP were 105%, 106%, and 93%, respectively. The reproducibility of the total procedure was tested using the sample of *Ephedra sinica* Stapf extracts. The relative standard deviations (RSDs) were 1.5%, 1.2%, and 2.0% (n = 4) for 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 ephedrine-containing medicinal herbs.

Because the peaks of NE and NPE were tailed and the contents of NE and NPE in *Ephedra sinica*

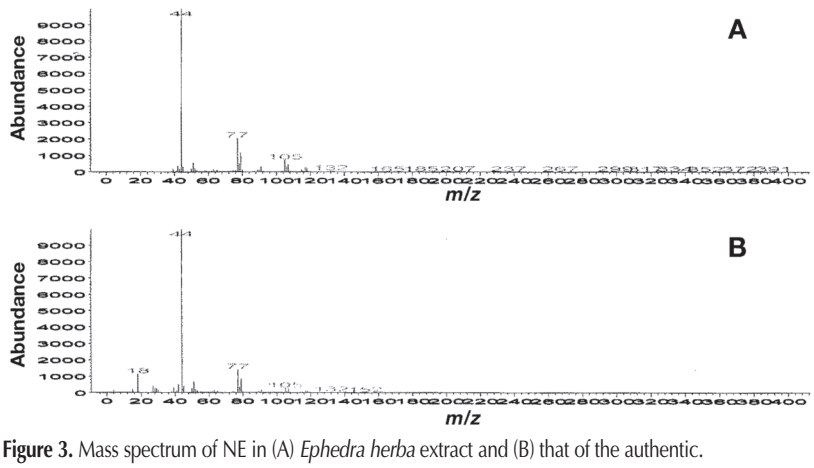

Figure 3. Mass spectrum of NE in (A) *Ephedra herba* extract and (B) that of the authentic.

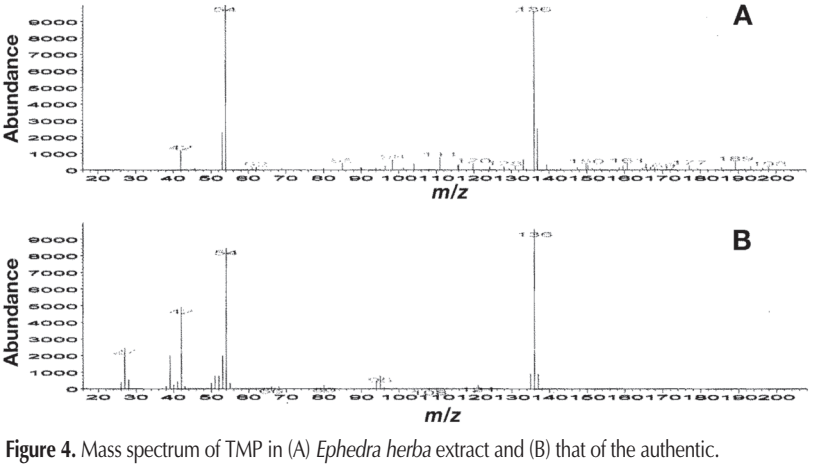

Figure 4. Mass spectrum of TMP in (A) *Ephedra herba* extract and (B) that of the authentic.

Stapf were very low, NE and NPE were not quantitated in this study.

Conclusion

Ephedrine alkaloids and TMP were simultaneously determined by GC–MS without tedious pretreatment, such as derivatization. Both the full-scan acquisition mode and SIM mode were used to identify and quantitate the alkaloids in *Ephedra sinica* Stapf. It was a simple, reliable, and quick method for the determination of the active components in ephedrine-containing medicinal herbs.

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