Quantitative Analysis of Ephedrine Analogues from *Ephedra* Species Using ^{1}H-NMR

Hye Kyong KIM, Young Hae CHOI, Wen-Te CHANG, and Robert VERPOORTE*

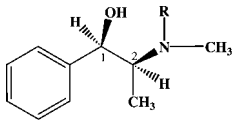
Division of Pharmacognosy, Institute of Biology, Leiden University; P. O. Box 9502, 2300 RA Leiden, The Netherlands.
Received July 28, 2003; accepted September 16, 2003

Four ephedrine analogues such as ephedrine, pseudoephedrine, methylephedrine, and methylpseudoephedrine were determined by ^{1}H-NMR from *Ephedra* species. In the region of δ 5.0—4.0, the signals of H-1 attached to the same carbon with a hydroxyl, were well separated from each other in $CDCl_3$. The amount of each alkaloid was calculated by the relative ratio of the intensity of H-1 signal to the known amount of internal standard, 200 μg of anthracene. This method allows rapid determination of the quantity of four ephedrine alkaloids from *Ephedra* species. The amount of these alkaloids was in the range of 1.0—2.0% of dry weight depending on the plant materials.

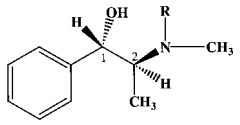
Key word *Ephedra*; quantitation; ^{1}H-NMR; ephedrine analogues

Ephedra, known as Ma-huang, is one of the oldest medicinal plants used in traditional Chinese medicine.[1] The genus of *Ephedra*, which belongs to the family of Ephedraceae, contains over 50 species. The aerial parts of *Ephedra* have long been used as a diaphoretic, anti-asthmatic and diuretic as well as for the treatment of bronchitis and acute nephritic edema.[2] The main active components are three pairs of optically active diastereomeric alkaloids[3]: (−)-ephedrine and (+)-pseudoephedrine, (−)-methylephedrine and (+)-methylpseudoephedrine, (−)-norephedrine and (+)-norpseudoephedrine (Fig. 1). The concentration of these benzylamine alkaloids varies from 0.02 to 3.40% in the aerial parts of the plant.[4] *Ephedra* plant materials used in oriental medicine show quite variable quality because a number of species comprise the source of the *Ephedra* on the market. Moreover the diverse geographical origins of the plants make the total content of main active alkaloids quite different from plant to plant. For this reason, it is necessary to determine the contents of *Ephedra* alkaloids in plant material. The methods for analysis of *Ephedra* alkaloids include thin layer chromatography,[5] gas chromatography,[6] isoachophoresis,[7] high performance liquid chromatography (HPLC)[8] capillary electrophoresis (CE).[9] Separation of these alkaloids is not possible with TLC and GC without derivatization. There is a report using CE in which the above mentioned alkaloids can be separated,[4] but up to now HPLC has been used most often for the ephedrine alkaloids analysis. HPLC, however, is not always satisfactory. In many cases sodium dodecyl sulfate (SDS) was used as a constituent of the mobile phase to increase the theoretical plate number and resolution, but separation of ephedrine and pseudoephedrine seems to be dependent on the brand and concentration of SDS.[10,11] The lack of specific and strong chromophore for detection is another problem when a conventional HPLC–UV detector is used. Moreover, HPLC and GC analysis required an elaborate clean up procedure and derivatization procedures in order to enhance sensitivity and to remove compounds that interfere with the detection of the target compounds. Therefore, an alternative method for the analysis of ephedrine alkaloids from *Ephedra* herbs would be highly desirable.

In this paper, we described the quantitative analysis of *Ephedra* alkaloids from *Ephedra* herbs using ^{1}H-NMR spectroscopy. It allows rapid and simultaneous determination of four ephedrine analogues ephedrine, pseudoephedrine, methylephedrine and methylpseudoephedrine without any pre-cleaning steps.



R = H, Ephedrine
R = CH_3, Methylephedrine



R = H, Peudoephedrine
R = CH_3, Methylpseudoephedrine

Fig. 1. Structures of Ephedrine Alkaloids

Experimental

Plant Material Nine *Ephedra* plant materials were purchased from a Taiwan market. *Ephedra sinica* and *Ephedra intermedia* were obtained from Taiwan Pharmaceutical Company (Sun Ten Phytotech Co., Taipei, Taiwan, R.O.C) and authentified by Prof. Y. S. Chang (Institute of Chinese Pharmaceutical Sciences, Chinese Medical University, Taichung, Taiwan, R.O.C).

Solvents and Chemicals First grade diethyl ether, chloroform, and methanol were purchased from Merck Biosolve Ltd. (Valkenswaard, The Netherlands). $CDCl_3$ (99.9%) was purchased from Cortec (Paris, France). Potassium hydroxide, anthracene, sodium chloride were obtained from Sigma (St Louis, MO, U.S.A.). (−)-Ephedrine, (+)-pseudoephedrine, (−)-methylephedrine and (+)-methylpseudoephedrine were purchased from Sigma (St Louis, MO, U.S.A.).

Extraction The extraction of alkaloids was performed according to the method of Cui *et al.*[12] Powdered plant material (200 mg) was weighed and transferred to 10 ml centrifuge tube and mixed with 8 ml of 0.5 M H_2SO_4 solution. The mixture was shaken and sonicated for 1 h and then centrifuged for 20 min at 3000 rpm. The supernatant layer was transferred to another tube and 1.4 ml of 5 M KOH solution, 2.4 g NaCl and 5 ml of diethyl ether were added to the tube. The mixture was shaken for 15 min and then centrifuged for 20 min at 3000 rpm. Extraction with diethyl ether was performed twice. Diethyl ether layers were combined into 20 ml round flask and evaporated after addition of internal standard. Dried sample was dissolved in 1 ml $CDCl_3$ and used for ^{1}H-NMR measurement.

* To whom correspondence should be addressed. e-mail: verpoort@LACDR.leidenuniv.nl

^{1}H-NMR Apparatus and Parameters ^{1}H-NMR spectra were recorded in 1 ml of $CDCl_3$ using a Bruker AV 400 spectrometer (equipped with an Indy Silicon graphics computer). For each sample, 128 scans were recorded with the following parameters: 0.17 Hz/point, pulse width (PW)=4.0 μs, and relaxation delay (RD)=1.0 s. FIDs were Fourier transformed with LB=0.30 Hz, GB=0 Hz, and PC=1.0. For quantitative analysis, peak area was used and the start and end point of the integration of each peak were selected manually.

Recovery Test of *Ephedra* Alkaloids One gram of filter paper disk (589[2] white, Schleicher & Schuell, GmbH, Cassel, Germany) was cut into *ca.* 1.5 cm diameter and placed in the extraction vessel. Each standard of ephedrine alkaloids (1 mg) was spiked into a filter paper disks. Then the spiked samples were dried in vacuum oven for 2 h.

Results and Discussion

For the quantitative analysis of the four alkaloids, ephedrine (EP), pseudoephedrine (PE), methylephedrine (ME), and methylpseudoephedrine (MP), H-1 was selected as a target signal in the ^{1}H-NMR spectra because these signals of the ephedrine analogues have separated resonances and do not overlap with other signals from the extract. These signals from the four compounds are well separated in the region of δ 4.0—5.0 when $CDCl_3$ is used, even though they are diastereomers (Fig. 2). The chemical shifts of H-1 of ephedrine and pseudoephedrine are δ 4.77 (d, J=3.9 Hz) and δ 4.17 (d, J=8.2 Hz), respectively. Another pair of diastereomers, methylephedrine and methylpseudoephedrine are also well separated from each other and show up at δ 4.96 (d, J=3.8 Hz) and δ 4.19 (d, J=6.4 Hz), respectively. In addition to their own chemical shift, the diastereomers can be differentiated by their coupling constants. The compounds which have (*S*,*S*) configuration show a larger coupling constant than those of a (*R*,*S*) configuration, because the dihedral angle between two protons at C-1 and C-2 is quite different. Therefore, it was found that the H-1 signal is most suitable to use as a target peak compared to the other signals which appeared in two other distinct regions: the *C*-methyl region (δ 0.6—1.0) and *N*-methyl region (δ 2.0—2.4). When we used CD_3OD as a solvent, the water peak from CD_3OD interfered with H-1 signals of ephedrine and methylephedrine and in the case of acetone-d_6, a broad –OH peak interferes to target signals in the region of δ 4.7—4.8. For these reasons, $CDCl_3$ is used.

Next, the calibration curve for each compound using the ratio of the peak area of the compound and the internal standard were determined in the range of 0.25—2.0 mg/ml in order to evaluate the accuracy of this method depending on the different concentrations. Each calibration curve is shown in Fig. 3. The linearity of ephedrine (EP), pseudoephedrine (PE), methylephedrine (ME) and methylpseudoephedrine (MP) were found to be higher than 0.990 (0.9994, 0.9980, 0.9965, 0.9989, respectively). Actually, these calibration curves for quantitation of the compounds are not really necessary for determination of alkaloids because the integration of the peaks is always proportional to the amount of the compound and the same for all compounds in ^{1}H-NMR.

For testing the recovery from a matrix, 1.0 mg of each compound was extracted from cellulose papers on which the compounds were adsorbed. The extraction method consists of a simple acid extraction, neutralization step, and liquid–liquid fractionation. Therefore as much as possible the extraction procedure from plant material was followed. The recovery of each compound with the chosen method was found

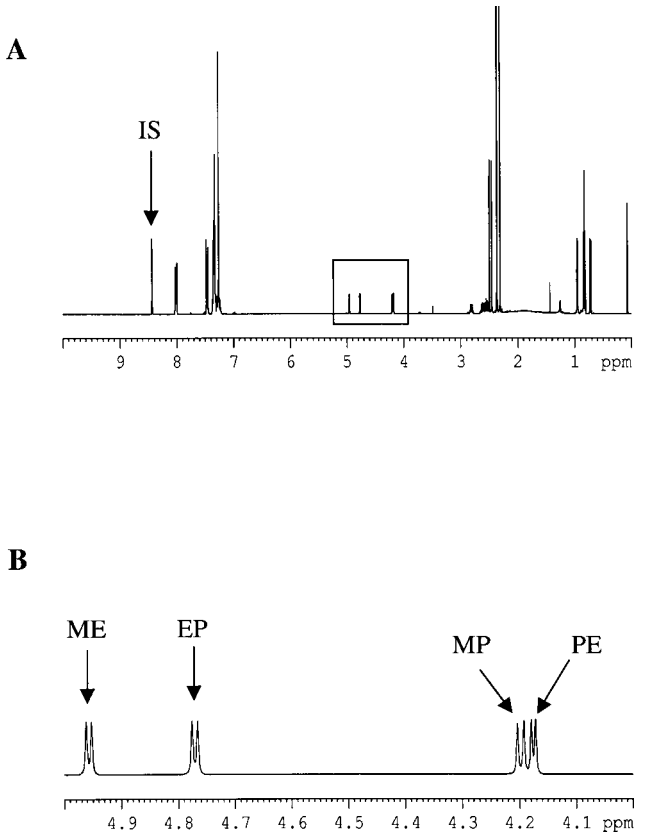


Fig. 2. ^{1}H-NMR Spectroscopy of Mixture of Four Ephedrine Alkaloids in $CDCl_3$ (400 MHz) after 128 Scans

(A) Range of δ 0.0—10.0. (B) Expansion in the range of δ 4.0—5.0. Arrows indicate H-1 proton of each compound, ME: methylephedrine, EP: ephedrine, MP: methylpseudoephedrine, PE: pseudoephedrine, IS: internal standard (anthracene).

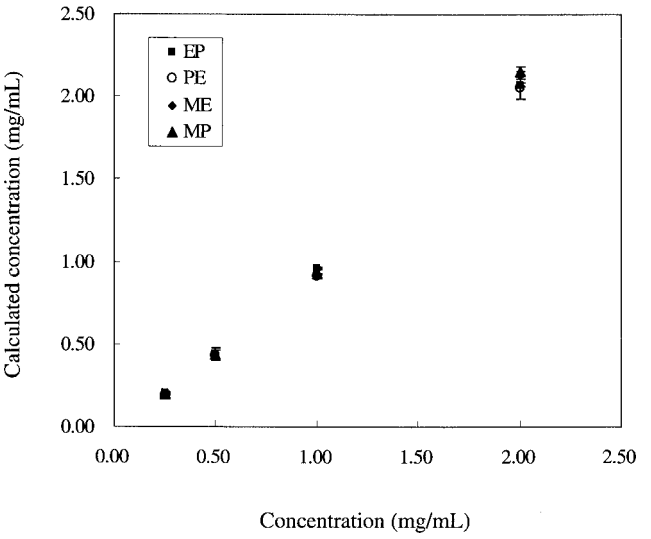


Fig. 3. Calibrations for Ephedrine Alkaloids Calculated from the Integral of H-1 Compared to Internal Standard (Anthracene)

ME: methylephedrine, EP: ephedrine, MP: methylpseudoephedrine, PE: pseudoephedrine.

to be more than 88%, EP: 90 (±1.6), PE: 88.3 (±2.1), ME: 92.9 (±2.1), MP: 91.9 (±1.9) percent, respectively.

Finally, *Ephedra* materials including *E. sinica* and *E. intermedia* were analyzed for ephedrine (EP), pseudoephedrine (PS), methylephedrine (ME), methylpseudoephedrine (MP)

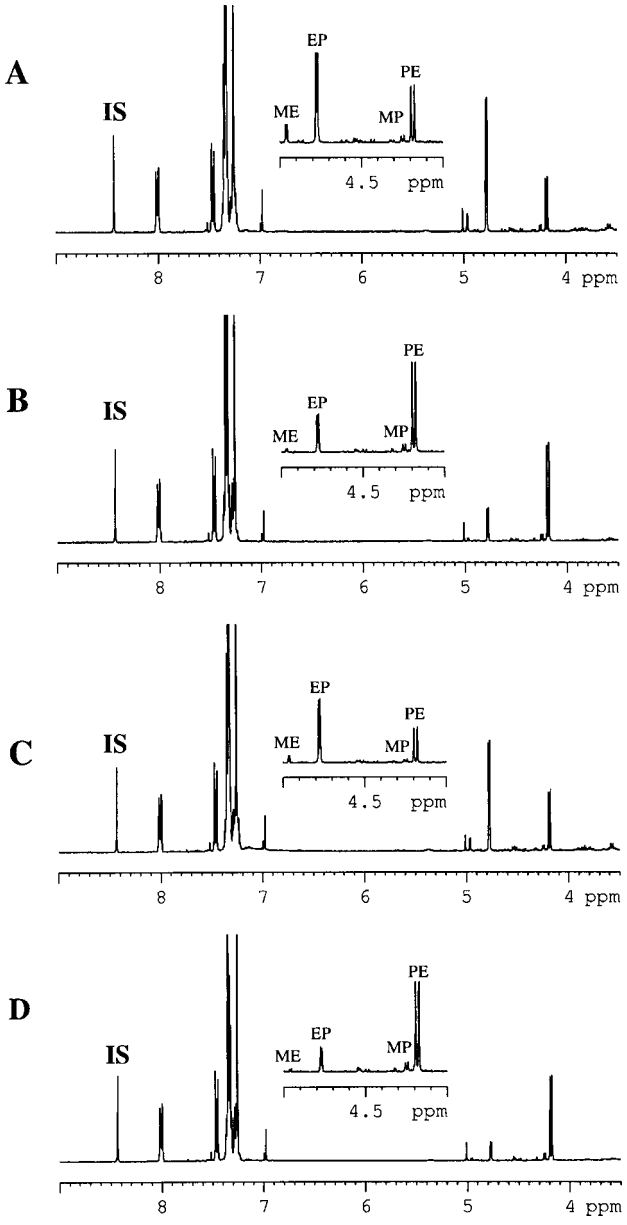


Fig. 4. ^{1}H-NMR Spectra of Alkaloid Fraction of *Ephedra* Herbs in the Range of δ 3.5—8.5

(A) *Ephedra sinica*, (B) *Ephedra intermedia*, (C) *Ephedra* sample No. 6, (D) *Ephedra* sample No. 2. Inset figures showed expansion of δ 4.0—5.0. ME: methylephedrine, EP: ephedrine, MP: methylpseudoephedrine, PE: pseudoephedrine. IS: internal standard (200 μg of anthracene).

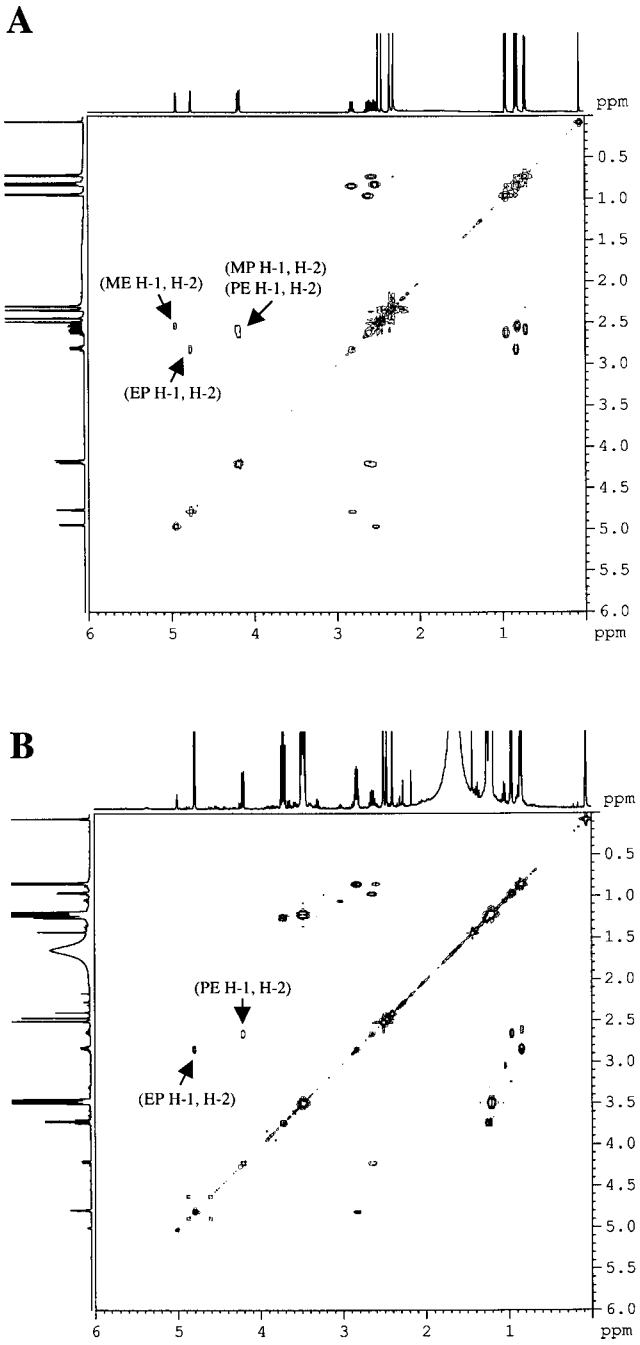


Fig. 5. ^{1}H–^{1}H COSY Spectra of the Mixture of Four Ephedrine Alkaloids (A) and *Ephedra sinica* (B) in the Range of δ 0.0—6.0.

using the ^{1}H-NMR method. For all samples tested in this study, the H-1 peak of each ephedrine alkaloid are quite well separated from the others and also no interference with other peaks was observed in the range of δ 4.0—5.0 in the ^{1}H-NMR spectrum (Fig. 4). This was confirmed by ^{1}H–^{1}H COSY spectra (Fig. 5). In this region the correlation between the H-1 signal and H-2 signal (δ 2.0—2.5, q) can be detected.

Table 1 shows the results of the quantitation of the alkaloids in the various plant materials. The results obtained by the ^{1}H-NMR method were found to be highly accurate with a standard deviation of about 5—10%. The content of the four alkaloids in the various samples were quite different, varying from 1 to 2% of dry weight. It is known that among *Ephedra* species only *Ephedra intermedia* has a lower amount of ephedrine than pseudoephedrine. Most of the samples evaluated in this study showed a lower ephedrine than pseudoephedrine content. The data from the ^{1}H-NMR are quite similar to the data from HPLC which were available from the Pharmaceutical Company, that provided these materials (Personal communication).

Using the described method, the contents of the ephedrine alkaloids can be analyzed within much shorter time than conventional chromatographic methods. Moreover, four different alkaloids can be detected without any derivatization. This ^{1}H-NMR method has the additional advantage that at the same time it is rapid, simple and an overall profile of the

Table 1. The Content of Ephedrine Alkaloids of Nine Different *Ephedra* Plants Including *Ephedra sinica* and *Ephedra intermedia*

Sample	Ephedrine (EP)	Pseudoephedrine (PE)	Methylephedrine (ME)	Methylpseudoephedrine (MP)	Total alkaloids[a] (%)
E. sinica	8.98±1.23	3.92±0.44	1.30±0.18	0.75±0.10	1.49
E. intermedia	2.15±0.31	6.29±0.87	0.22±0.04	0.76±0.14	0.94
1	2.61±0.15	9.44±0.85	0.38±0.03	1.48±0.07	1.39
2	1.57±0.29	6.99±1.01	0.20±0.04	1.07±0.22	0.98
3	2.84±0.14	8.22±0.46	0.39±0.02	1.07±0.07	1.25
4	1.87±0.18	8.70±0.99	0.26±0.08	1.27±0.27	1.21
5	1.59±0.09	8.63±0.55	0.21±0.03	1.25±0.15	1.17
6	9.12±0.45	5.09±0.17	1.10±0.03	0.91±0.02	1.62
7	1.69±0.11	9.06±0.53	0.17±0.04	1.49±0.09	1.24
8	1.64±0.16	8.73±0.95	0.23±0.04	1.06±0.02	1.17
9	2.23±0.04	9.88±0.23	0.32±0.02	1.43±0.14	1.39

The amounts were calculated from the peak area of H-1 compared to that of 200 μg of anthracene (mg/g dry weight±S.D.). All experiments were done in triplicate. *a*) Sum of four alkaloids (EP, PE, ME, MP) analyzed in this study was expressed in percentage of 1 g of dry weight.

preparation can be obtained. This can be applied to the analysis of commercial pharmaceutics or products of *Ephedra* such as cough syrups and dietary supplements.

Acknowledgement The authors would like to thank W. C. Chung (Sun Ten Phytotech Co., Taipei, Taiwan, R.O.C) for a donation of two authentic plant materials. The Post-doctoral Fellowship Programs of Korea Science & Engineering Foundation (KOSEF) are gratefully acknowledged for their support to H. K. Kim and Y. H. Choi. We are also grateful for the financial support of the van Leersumfonds (KNAW).

References

1) Nagai W. N., Kanao S., *J. Pharm. Soc. Jpn.*, **48**, 845—851 (1928).
2) Tang W., Eisenbrand G., "Chinese Drugs of Plant Origin," Springer Verlag, New York, 1992, p. 481.
3) Leung A. Y., Foster S., "Encyclopedia of Common Natural Ingredients," 2nd ed., John Wiley & Sons, New York, 1996.
4) Liu Y.-M., Sheu S.-J., Chiou S.-H., Chang H.-C., Chen Y.-P., *Planta Med.*, **59**, 376—377 (1993).
5) Hashimoto Y., Ikeshiro Y., Higashiyama T., Audo K., Endo M., *Yakugaku Zasshi*, **97**, 594—596 (1977).
6) Yamasaki K., Fujita K., Okada K., Yoshida M., Tanaka O., *Chem. Pharm. Bull.*, **22**, 289—292 (1974).
7) Kasahara Y., Hikino H., *J. Chromatogr.*, **324**, 503—507 (1985).
8) Zhang J., Tian Z., Lou Z., *Planta Med.*, **54**, 69—70 (1988).
9) Liu Y.-M., Sheu S.-J., *J. Chromatogr.*, **600**, 370—372 (1992).
10) Okamura N., Miki H., Harada T., Yamashita S., Masaoka Y., Nakamoto Y., Tsuguma M., Yoshitomi H., Yagi A., *J. Pharm. Biomed. Anal.*, **20**, 363—372 (1999).
11) Sheu S. J., Huang M.-H., *J. Food Drug Anal.*, **8**, 337—341 (2000).
12) Cui J.-F., Zhou T.-H., Zhang J.-S., Lou Z.-C., *Phytochem. Anal.*, **2**, 116—119 (1991).