Note

Determination of ephedrine alkaloids by isotachophoresis*

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As a result of pharmacological investigations of the *Ephedra* herb, it has been shown that most of the clinical efficacy is exerted by its alkaloidal components, which include (−)-ephedrine (E), (+)-pseudoephedrine (PE), (−)-methylephedrine (ME), (+)-methylpseudoephedrine (MPE), (−)-norephedrine (NE) and (+)-norpseudoephedrine (NPE).

Several methods have been reported for the simultaneous determination of some of these six alkaloids: *i.e.* salting out chromatography[1], thin-layer chromatography[2,3], gas–liquid chromatography[4] and high-performance liquid chromatography[5]. However, the separation of all these alkaloids by these methods is unknown. Furthermore, in the case of the analysis of the ephedrine alkaloids in the crude drug, *Ephedra* herb, the known methods require tedious pretreatments of *Ephedra* extracts before analysis, leading to possible sources of error.

Isotachophoresis (ITP) has already been successfully applied to the analysis of ionic components contaminated with other components, and which require simple pretreatments of samples before analysis because no absorbents are employed in the separation tubes. We have thus examined the utility of ITP for the simultaneous determination of all the six ephedrine alkaloids. This paper describes the development of a suitable method for the analysis of the ephedrine alkaloids and its application to analysis of the crude drug, *Ephedra* herb.

EXPERIMENTAL

Reagents and materials

Barium hydroxide, *β*-alanine, histidine, polyvinyl alcohol (polymerization degree 2000) (PVA), 2-amino-2-methyl-1,3-propanediol (Ammediol) and Triton X-100 were obtained from Wako Pure Chemicals (Osaka, Japan) and hydroxyethyl cellulose (HEC) (Shimadzu IP-Kit, X-200) from Shimadzu (Kyoto, Japan).

Ephedrine (E) hydrochloride and norephedrine (NE) hydrochloride were purchased from Dainippon Pharmaceutical (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan), respectively. Hydrochlorides of pseudophedrine (PE), methylephedrine (ME), methylpseudoephedrine (MPE) and norpseudoephedrine (NPE) were prepared at Tohoku University. An *Ephedra* herb was obtained from the crude drug market (Osaka, Japan).

Preparation of extracts of Ephedra herbs

Ephedra herb was pulverized and dried in a desiccator. The powdered sample (2.0 g) was vigorously shaken with 50% ethanol (80.0 ml) for 30 min at room temperature, then centrifuged at 1500 *g* for 10 min. The supernatant (40.0 ml) was mixed with 0.1 *M* hydrochloric acid (1.0 ml) and evaporated to give the *Ephedra* extract.

Apparatus and procedure

All analyses were carried out on a Shimadzu Model IP-2A isotachophoretic analyzer equipped with a Shimadzu potential gradient detector with a 40-mm PTFE capillary (1.0 mm I.D.) as the pre-column and a 150-mm FEP capillary (0.5 mm I.D.) connected in series. The leading electrolyte was prepared by adding β-alanine or histidine to 0.005 *M* barium hydroxide to adjust the pH. 0.1% PVA, HEC or Triton X-100 was added to improve separation by the enhancement of viscosity and reduction of electroendosmosis. 0.01 *M* Ammediol was used as the terminating electrolyte. The analysis first proceeded at 200 μA for 12 min and was then continued at 100 μA.

Analysis of ephedrine alkaloids in Ephedra extract

Calibration curves (step lengths *vs.* amounts injected) for the six ephedrine alkaloids were constructed in the range 0.2–2.0 mg/ml. The *Ephedra* extract (20.0 mg) was dissolved in water (1.0 ml) and an aliquot (0.010 ml) was injected directly.

RESULTS AND DISCUSSION

Examination of analysis conditions

In general, the dissociation of weakly acidic and weakly alkaline ionic substances is affected by the pH of a solution. Therefore, in electrophoresis, the mobility of ionic substances is greatly influenced by pH. In the case of ITP, because the sample ions are present in a leading electrolyte, their mobilities can change remarkably with the pH of the leading electrolyte. For the six E alkaloids which migrate as cations, it was expected that their mobilities would not differ very much at low pH because of excessive dissociation. Hence, it was decided to use a leading electrolyte of higher pH. Barium hydroxide was chosen since it is alkaline and migrates well. Ammediol was selected as a terminating electrolyte because its mobility is lower than those of the sample alkaloids.

Kinoshita *et al.*[6] investigated the conditions for separation of E, PE and ME by ITP utilizing barium ion as the leading ion, glutamic acid or glutamine as a buffer ion (counter ion) of the leading electrolyte and Tris–hydrochloric acid or betaine–hydrochloric acid as a terminating electrolyte. Nishimura *et al.*[7] also tried to analyze E, PE and ME by ITP employing barium ion as the leading ion with β-alanine as a

counter ion and Tris–hydrochloric acid as a terminating electrolyte. Although E, PE and ME were separated under those conditions, the other three analogues, MPE, NE and NPE, were not. Analysis of the six E analogues was thus carried out in this work by referring to those conditions.

When sample ions migrate in a capillary tube, the diffusion in the leading electrolyte, convection by Joule heat and electroosmosis by the ζ-potential generated by interaction of sample ions with the capillary tube sometimes cause disturbance of the zone boundaries between ionic components, resulting in insufficient separation. In order to prevent those phenomena, a surface active agent or a polymer of the cellulose or polyalcohol types is usually added to the leading electrolyte. Thus, separation of the six E alkaloids was examined under conditions where 0.1% Triton X-100, HEC or PVA was added to the leading electrolyte which comprised 0.005 M barium hydroxide with β-alanine as a counter ion and adjusted to pH 10.5. As a terminating electrolyte, 0.01 M Ammediol was selected. It was found that in the absence of an additive or in the presence of Triton X-100, the E alkaloids did not migrate with well separated zone boundaries. Although with the addition of HEC the separation was slightly improved, the sharpness of the zone boundaries was still insufficient. After the addition of PVA, the six alkaloids were detected as five stepwise signals. Hence, PVA was used as the additive to the leading electrolyte in subsequent experiments. However, because two of the analogues, ME and NPE, could not be separated, the effects of the pH of the leading electrolyte and counter ions were investigated. When β-alanine or histidine was the counter ion at pH 9.5, 10.0, 10.5 or 11.0 the alkaloids were separated but ME and NPE were still overlapped. At pH 9.5 and 10.0 the mobilities of the alkaloids were similar, and at pH 11.0, disturbance of the signals of the potential gradient was observed. Therefore, β-alanine was selected as a counter ion and the pH adjusted to 10.5, the conditions which gave better separations.

In ITP, the addition of organic solvents such as methanol, ethanol and acetone to water as a leading electrolyte is known to alter the mobilities of ions. In order to separate ME and NPE, ITP was performed in a leading electrolyte prepared by mixing 0.005 M barium hydroxide, adjusted to pH 10.5 by β-alanine and containing 0.1% PVA, and methanol in the ratio 4:1, and with 0.01 M Ammediol as terminating electrolyte. This resulted in a complete separation of all the six E alkaloids (Fig. 1) (potential unit values[8]: E, 0.47; PE, 0.56; ME, 0.72; MPE, 0.32; NE, 0.95; NPE, 0.77).

Calibration curves for ephedrine alkaloids

Calibration graphs (step lengths *vs.* amounts analyzed) were constructed and found to be linear. The equations for the E analogues were as follows: E, $y = 12.9x + 0.3$ ($r = 0.999$); PE, $y = 9.1x + 0.4$ ($r = 0.999$); ME, $y = 9.4x - 0.5$ ($r = 0.999$); MPE, $y = 12.3x - 0.1$ ($r = 0.999$); NE, $y = 9.5x + 0.3$ ($r = 0.998$); NPE, $y = 7.9 x - 0.2$ ($r = 0.998$).

Determination of ephedrine alkaloids in Ephedra herb

When the *Ephedra* extract was subjected to ITP under the selected conditions, the isotachopherogram shown in Fig. 2 was obtained. The zones were identified by comparison of the step heights of the sample components with those of the standard
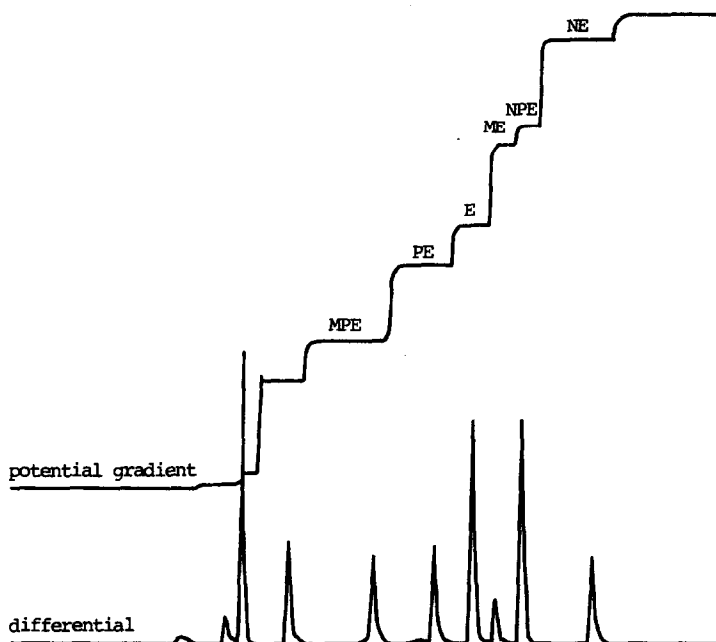
Fig. 1. Isotachophoretic separation of a mixture of ephedrine alkaloids. E = Ephedrine; PE = pseudo-ephedrine; ME = methylephedrine; MPE = methylpseudoephedrine; NE = norephedrine; NPE = nor-pseudoephedrine.
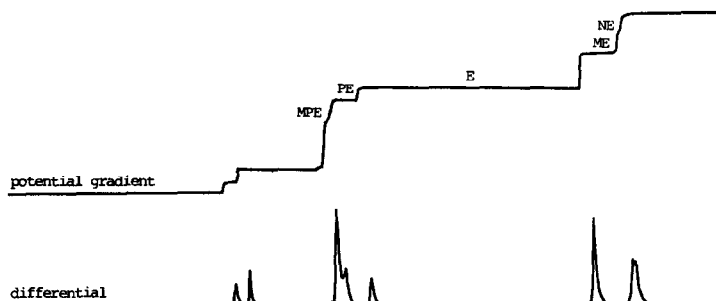

Fig. 2. Isotachopherogram of an *Ephedra* extract. For abbreviations, see Fig. 1.

alkaloids. Thus the following alkaloids were identified in the analyzed sample: E, PE, ME, MPE and NE. Substitution of the lengths of potential gradient zones for x in the above derived equations afforded the contents of the E analogues in the *Ephedra* herb (%, $n = 3$): E, 0.733 ± 0.073; PE, 0.129 ± 0.031; ME, 0.114 ± 0.014; MPE, 0.009 ± 0.000; NE, 0.014 ± 0.004.

From the results it can be concluded that simultaneous determination of the E alkaloids in *Ephedra* herbs by means of ITP has some advantages: small sample size, no tedious pretreatment, short analysis time and simple quantification.

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