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Determination of ephedrine compounds in nutritional supplements by cyclodextrin-modified capillary electrophoresis

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Abstract

Capillary electrophoresis was utilized for the separation, identification, and quantitation of ten stereoisomers in the ephedrine family. Chiral discrimination was accomplished through the use of hydroxypropyl-β-cyclodextrin, and separation was enhanced at pH 2 in the presence of tetramethylammonium chloride and sodium dodecyl sulfate. Calibration plots of the ephedrines were linear over the range 4–100 μg/ml. This method was used in the analysis of nutritional supplements that contain Ma Huang, a Chinese herbal preparation that is made from plants in the genus *Ephedra*.

1. Introduction

Ma Huang is a Chinese herbal preparation that is obtained from plants of the genus *Ephedra*. The active principle components are alkaloids, among which (−)-ephedrine can account for 80 to 90%; the others may include (+)-pseudoephedrine, (−)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, (−)-norpseudoephedrine and (−)-norephedrine [1,2]. Although the U.S. Pharmacopeia gives methods for the determination and quantitation of ephedrine and pseudoephedrine, no distinction among stereoisomers is possible for unknown substances using current protocols.

Samples of an "all natural nutritional supplement" reportedly containing Ma Huang were received at the National Forensic Chemistry Center for the determination of the presence of amphetamines and/or amphetamine precursors, e.g. the ephedrines. If ephedrines were detected, we were requested to establish whether they came from natural or synthetic sources. This question requires the separation and identification of the (+)- and (−)- enantiomers.

Capillary electrophoresis (CE) has become an important tool in the analysis of chiral compounds, particularly in the determination of optical purity. Preparations containing *Ephedrae herba* have been studied by CE for the visualization of the six alkaloids [3] and for the quantitation of ephedrine and pseudoephedrine in various Chinese herbal preparations [4]. However, resolution of enantiomers is not possible using these methods.

Derivatization of (±)-ephedrine, (±)-pseudoephedrine, (±)-norephedrine, and (±)-norpseudoephedrine with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) led to enantiomeric separations under micellar electrokinetic capillary chromatographic conditions [5]. Additionally, chiral separations can be ac-

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complished by incorporating cyclodextrins as chirally active buffer components. Previous work with ephedrines [6–9] suggested that these separations would be more effective if chemically modified β-cyclodextrins were used. Resolution was improved further by decreasing or eliminating electroosmotic flow (EOF), either at low pH [6–9], or upon the addition of a cationic species such as tetramethylammonium chloride (TMAC) [9].

The CE method presented here utilizes hydroxypropyl-β-cyclodextrin as the chiral selector at low pH with TMAC added to further decrease the EOF. This buffer system permits at least partial resolution of the stereoisomers of ten members of the ephedrine family, and the separation of potential derivatives D-amphetamine, L-amphetamine, D-methamphetamine, and L-methamphetamine from their precursors.

2. Experimental

2.1. Instrumentation

All experiments were performed on an ISCO Model 3140 capillary electrophoresis system (ISCO, Lincoln, NE, USA), and utilized ICE data management and control software for data collection and manipulation. Solute peak areas were normalized with respect to their migration times during data processing.

2.2. Materials

The standards utilized in these studies were purchased from Sigma (St. Louis, MO, USA), and are listed in Table 1 with product name, common name of the free base, and the abbreviations used. Hydroxypropyl-β-cyclodextrin, HP-β-CD (average molar substitution 0.8; average molecular mass 1500) was obtained from Aldrich (Milwaukee, WI, USA). Heptakis(2,6-di-O-methyl)-β-cyclodextrin (Heptakis) was purchased from Sigma. Tetramethylammonium chloride (TMAC), also from Sigma, was supplied as a 5 *M* solution. All other chemicals were reagent grade. Distilled, deionized water (DDW) was obtained in the laboratory from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). DDW was acidified to pH 2.0 with concentrated phosphoric acid. Buffers were filtered through 0.2-μm Nylon 66

Table 1

Standards used in these studies

Product name	Common name, free base	Abbreviation
(1*R*,2*S*)-(−)-Ephedrine hemisulfate	l-Ephedrine	(−)-E
(1*S*,2*R*)-(+)-Ephedrine hemihydrate	d-Ephedrine	(+)-E
(1*R*,2*R*)-(−)-ψ-Ephedrine	l-Pseudoephedrine	(−)-ψE
(1*S*,2*S*)-(+)-ψ-Ephedrine hemisulfate	d-Pseudoephedrine	(+)-ψE
(1*R*,2*R*)-(−)-N-Methyl-ψ-ephedrine	(−)-N-Methylpseudoephedrine	(−)-Me-ψ-E
(1*S*,2*S*)-(+)-N-Methyl-ψ-ephedrine	(+)-N-Methylpseudoephedrine	(+)-Me-ψ-E
(1*R*,2*S*)-(−)-N-Methylephedrine	(−)-N-Methylephedrine	(−)-MeE
(1*S*,2*R*)-(+)-N-Methylephedrine	(+)-N-Methylephedrine	(+)-MeE
(−)-Norpseudoephedrine hydrochloride	(−)-Norpseudoephedrine	(−)-Nor-ψ-E
(1*R*,2*S*)-(−)-Phenylpropanolamine	(−)-Norephedrine	(−)-Nor
(1*S*,2*R*)-(+)-Phenylpropanolamine	(+)-Norephedrine	(+)-Nor
d-Amphetamine sulfate	D-Amphetamine	D-Amph
d,l-Amphetamine sulfate	D,L-Amphetamine	D,L-Amph
(+)-Methamphetamine hydrochloride	D-Methamphetamine	D-Meth
(−)-Deoxyephedrine[a]	L-Methamphetamine	L-Meth

[a] Supplied as a 1.0 mg/ml standard solution in methanol.

filters and were degassed under aspirator vacuum.

2.3. Standard preparation

Approximately 10 mg of each standard were dissolved in a sufficient volume of pH 2.0 DDW to prepare either 8.0 or 4.0 mg/ml free base stock solutions. These stock solutions were diluted with pH 2 DDW to prepare 0.40 mg/ml free base working solutions. Standards and mixtures for injection were prepared from either the stock or working solutions. Solutions were stored at 4°C. Calibration plots utilized standard concentrations of 4, 10, 20, 40, 60, and 100 μg/ml free base.

2.4. Sample preparation

The three nutritional supplements, called samples 1, 2, and 3, consisted of capsules that contained a brown powder. Capsules were emptied and a composite of the capsule contents of each sample was produced. Approximately 30 mg of composite were weighed into a glass vial, and 3.0 ml of pH 2.0 DDW were added to produce a 1:100 extraction solution. The vial was sonicated for 30 min, and the contents were filtered through a 0.2-μm Nylon 66 syringe filter. All solutions were stored at 4°C.

2.5. Sample analysis

Separations were accomplished in 90 cm × 50 μm I.D. uncoated fused-silica capillaries (65 cm to detector), using 70 m*M* HP-β-CD–30 m*M* TMAC–10 m*M* sodium dodecyl sulfate (SDS), pH 2.0 buffer. The running voltage was +28 kV at a temperature of 31°C. Samples were introduced using vacuum injection at 25.0 kPa s. Solutes were detected at 210 nm. The capillary was rinsed with buffer for 15 min at the start of the day, and for 4 min before each analysis.

Verification of the presence of (−)norephedrine made use of the buffer system developed by Nielen [8]. A 30 m*M* Tris-base stock solution was prepared in DDW and was acidified to pH 2.4 with concentrated phosphoric acid. Separations of ephedrines occurred in 30 m*M* Heptakis–30 m*M* Tris–H_3PO_4, pH 2.4 under the instrumental conditions used in the HP-β-CD buffer system.

The components present in the nutritional supplements were identified tentatively by comparing the peak migration times to those of the ephedrines contained in the standard mixture. Specific isomer identification and quantitation were aided by the addition of the appropriate standard(s) to the sample extracts. When necessary, the extracts were diluted to ensure the peak responses were within the linear dynamic range.

3. Results and discussion

The initial experiments performed in this laboratory utilized heptakis(2,6-di-O-methyl)-β-CD with Tris–H_3PO_4, pH 2 as the background electrolyte [8]. The six alkaloids found in Ma Huang were visualized in 14 min, but a ten-component mixture of the enantiomers yielded only eight peaks. Although this buffer system could have been manipulated to improve resolution, it was decided that the use of Heptakis was not cost effective for routine sample analyses. Additional experiments were performed using methyl-, hydroxyethyl-, and hydroxypropyl-substituted β-CDs. The results indicated that the hydroxypropyl derivative was the most promising for enantiomer resolution.

During optimization experiments, the concentration of HP-β-CD was increased, with a resulting increase in solute migration times and an improvement in resolution. The background electrolyte was changed from Tris–H_3PO_4 [8] to TMAC [9] in an effort to improve resolution further by decreasing the EOF. Addition of SDS improved resolution between (+)-ψE and (+)-Me-ψ-E. A coated glycerol capillary was evaluated, but no additional improvement in resolution was seen.

Fig. 1 shows an electropherogram obtained from a 20 μg/ml free base standard mixture, and Table 2 summarizes the average values for migration times obtained for each peak. Efficiencies for the solute peaks averaged 154 000 plates. Under these buffer conditions, (+)-Nor and (−)-Nor co-migrate as peak 2. Baseline res-

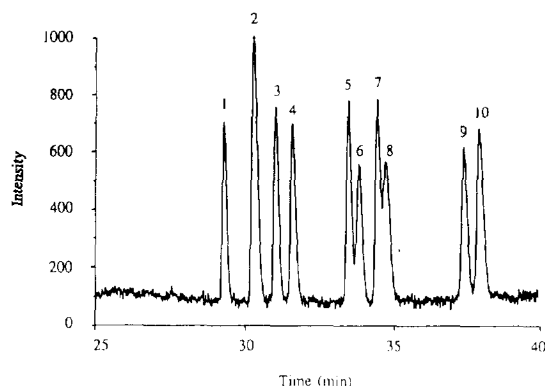

Fig. 1. CE analysis of a mixture of eleven ephedrine enantiomers, approximately 20 μg free base/ml each. Migration times and peak identifications are given in Table 2.

olution was not achieved for (±)-E (peaks 5 and 6) or (±)-MeE (peaks 7 and 8), and at solute concentrations above 20 μg/ml, resolution deteriorated rapidly. Although the resolution was essentially baseline between (+)-ψE (peak 9) and (+)-Me-ψ-E (peak 10) at 40 μg/ml, it decreased to a value of 1.0 at solute concentrations of 100 μg/ml.

All solutes displayed similar limits of detection of approximately 4 μg/ml for each free base. The calibration standards ranged from 4 to 100 μg/ml, and the solutes displayed linearity with correlation coefficients between 0.9797 for (+)-MeE and 0.9998 for (−)-ψE. Calibrations for (−)-E and (−)-MeE were verified by analysis in the absence of their enantiomers. A log–log plot of the concentration versus peak area for (−)-E yielded a slope of 0.9958.

Table 2
Migration data for ephedrines in the HP-β-CD buffer system

Compound	Peak	Migration time (min)	
		Standard[a]	Sample
(−)-Nor-ψ-E	1	29.5 ± 0.2	
(+)-Nor	2	30.5 ± 0.2	
(−)-Nor	2		30.7
(−)-ψ-E	3	31.3 ± 0.2	
(−)-Me-ψ-E	4	31.8 ± 0.2	
(+)-E	5	33.7 ± 0.2	
(−)-E	6	34.1 ± 0.2	33.5
(+)-MeE	7	34.6 ± 0.3	
(−)-MeE	8	35.0 ± 0.3	35.1
(+)-ψ-E	9	37.7 ± 0.3	37.5
(+)-Me-ψ-E	10	38.2 ± 0.3	

[a] $n = 4$.

Fig. 2 is a profile of a 1:100 extract of sample 1 of the nutritional supplements. As preliminary identification of ephedrine(s) in the nutritional supplements, the migration times of the components in the samples were compared to those in the standard mixture. Table 2 summarizes the average values for migration times obtained for each peak in sample 1. A concentration effect on the migration time of the peak identified as (−)-E is apparent. When the extract was diluted from 100 μl to 400 μl with pH 2.0 DDW, the peak identified as (−)-E migrated in 34.1 min, yielding a closer match to the standard.

To facilitate the identification and quantitation of the components in the acidic extracts, the addition of specific enantiomers to the sample extracts was correlated with the known components of Ma Huang [1–3]. This procedure was necessary due to the proximity of the migration of the solutes, as can be seen in Fig. 1 and Table 2.

Peak-area responses were used for quantitation by the ratio:

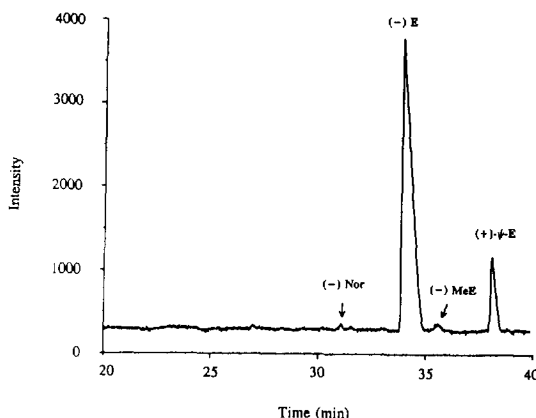

Fig. 2. CE analysis of a 1:100 acidic extract of sample 1 of the nutritional supplements. Migration times and peak identifications are given in Table 2.

$$\frac{\mu\text{g free base/ml solute}}{\text{peak area, solute}} = \frac{\mu\text{g free base/ml solute + std}}{\text{peak area, solute + std}}$$

The concentrations of (−)-ephedrine, (+)-pseudoephedrine, (−)-*N*-methylephedrine, and (−)-norephedrine in sample 1 are given in Table 3 for three determinations. The concentrations of the components may represent a dependence on the age of the solution. Extract 1 was prepared four days prior to analysis, and extracts 2 and 3 were prepared one to two days prior to analysis. Although (+)- and (−)-norephedrine co-migrate under these buffer conditions, the peak in Fig. 2 was tentatively identified as (−)-Nor. This was verified in subsequent experiments (discussed below).

Fig. 3 shows profiles of 1:100 extracts of the three supplements, as well as the free base standard components (lower trace). The concentrations of the ephedrines found in samples 1, 2 and 3 are given in Table 4. The primary component is (−)-E as expected, but it is not known whether the differences in component concentrations among the samples are significant. The relative amount of (−)-E varies with the species within the genus, and can account for 30–90% of the alkaloid content [10]; the quantity of (−)-E comprises approximately 50% of the alkaloids in *E. gerardiana* [2], and 85% in *E. sinica* [11]. The total alkaloid content and relative composition also varies with the time of the year and the geographic region in which the plant was harvested, as well as the climatic conditions during the growing season [11].

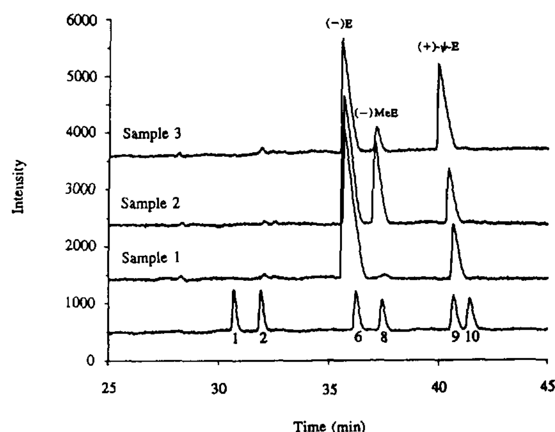

Fig. 3. CE analysis of the 1:100 acidic extracts of the three nutritional supplement samples, and the six free base standard components (lower trace). Standards are 20 μg/ml each, and are identified by numbers in Table 2.

Enantiomeric purity may be a consideration in determining whether the source of these alkaloids was natural or synthetic. Fig. 4 shows 10% (w/w) impurity levels of (+)-E and (+)-MeE with respect to their enantiomers in nutritional supplement sample 2, with poor resolution. Baseline resolution of both (±)-E [6,8] and (±)-MeE [8] is possible in buffer systems utilizing Heptakis. This system allows the separation of the norephedrine enantiomers, which co-migrate in a β-CD buffer [9] and in the present HP-β-CD buffer.

Additional experiments utilized 30 m*M* Heptakis–30 m*M* Tris–H_3PO_4, pH 2.4 [8] to

Table 3
Method reproducibility using sample 1

Compound	Concentration free base (mg/g)			Composition[a] (%)		
	Extr. 1	Extr. 2	Extr. 3	Extr. 1	Extr. 2	Extr. 3
(−)-Ephedrine	17.3	27.5	23.6	78.3	85.9	85.0
(+)-Pseudoephedrine	4.25	3.74	3.42	19.2	11.7	12.3
(−)-*N*-Methylephedrine	0.39	0.44	0.46	1.8	1.4	1.7
(−)-Norephedrine	0.16	0.33	0.29	0.7	1.0	1.0

[a] Calculated on the basis of total alkaloid concentration.

Table 4
Quantitation of ephedrines in three samples of nutritional supplement

Compound	Concentration free base (mg/g)			Composition[a] (%)		
	1	2	3	1	2	3
(−)-Ephedrine	22.8	12.8	8.9	84	51	49
(+)-Pseudoephedrine	3.8	4.4	7.7	14	17	42
(−)-*N*-Methylephedrine	0.43	8.1	1.6	1.6	32	8.8

[a] Calculated on the basis of total alkaloid concentration.

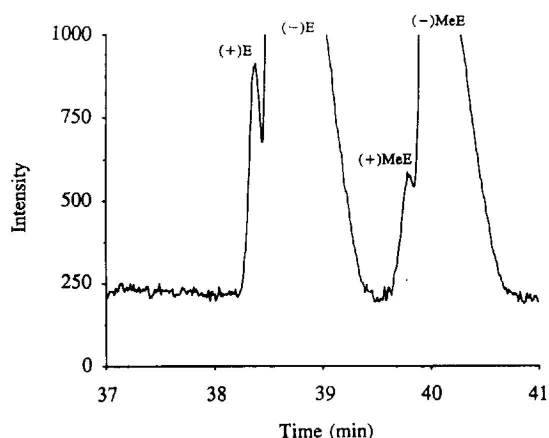

Fig. 4. CE analysis of sample 2 extract with 10% (w/w) (+)-E and (+)-MeE spikes with respect to their enantiomers.

verify the identification of norephedrine as the (−)-enantiomer. Average migration times for (±)-Nor and (±)-E standards are given in Table 5, and baseline resolution was achieved under these experimental conditions. Nielen [8]

Table 5
Migration data for norephedrine and ephedrine in the Heptakis buffer system

Compound	Migration time (min)[a]
(−)-Nor	16.08 ± 0.08
(+)-Nor	16.52 ± 0.08
(−)-E	17.32 ± 0.08
(+)-E	17.63 ± 0.09

[a] $n = 4$.

reported that, although (+)-MeE and (−)-MeE could be resolved from each other and from (±)-Nor, partial overlap occurred between (−)-MeE and (+)-E. This was verified in the present system when a (−)-MeE standard yielded a migration time of 17.50 min.

When samples 2 and 3 were injected, (−)-MeE migrated as a peak on the trailing edge of the (−)-E main peak, as expected. It remained possible to identify (−)-Nor by the appearance of peaks at 16.07, 16.04, and 16.05 min in samples 1, 2, and 3, respectively. Further verification was accomplished through the addition of a (−)-Nor spike to the extracts. These results suggested that neither the HP-β-CD nor the Heptakis buffer system alone was sufficient to answer all the questions concerning the samples. However, based on the combination of the data obtained from the two systems, it was determined that the ephedrines present in these nutritional supplements came from natural sources.

The other question about the product concerned the presence of amphetamines. Methamphetamine can be produced from either ephedrine or pseudoephedrine, and amphetamine can be produced from norephedrine and norpseudoephedrine. Within the HP-β-CD buffer system, the migration times of L-Amph (35.6 min), D-Amph (36.7 min), L-Meth (38.3 min) and D-Meth (40.0 min) standards yield similar standard deviations as the ephedrines listed in Table 2. When comparing the migration times of the amphetamines to the ephedrines, it is apparent that L-Amph migrates on the trailing edge of

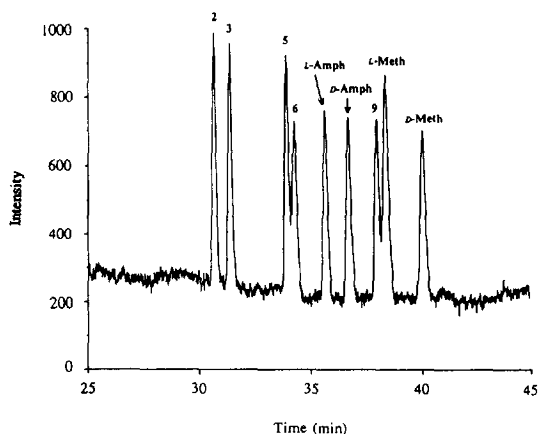

Fig. 5. CE analysis of a mixture of five ephedrine and four amphetamine standards, approximately 20 μg free base/ml each. The numbered peaks are identified in Table 2.

(−)-MeE, and L-Meth co-migrates with (+)-Me-ψ-E. (−)-Nor-ψ-E was not included because it migrates prior to (±)-Nor (see Fig. 1 and Table 2); a standard for (+)-Nor-ψ-E was not available, so its electrophoretic behavior under these conditions is unknown. However, Fig. 5 demonstrates the ability to separate the amphetamine enantiomers from most of their potential precursors. An interesting development of this method is that D- and L-amphetamine can be resolved in the presence of HP-β-CD, in contrast to (+)- and (−)-norephedrine. By comparing the separation of the standards in Fig. 5 to the profiles of the sample extracts in Fig. 3, it is apparent that no amphetamines are present in the nutritional supplements at detectable levels.

4. Conclusions

The utilization of a hydroxypropyl-modified β-cyclodextrin as a chiral discriminator has permitted the separation and identification of ten enantiomers in the ephedrine family by capillary electrophoresis. This method has been expanded to include the study of nutritional supplements that contain Ma Huang. When the samples were analyzed in this system and in a buffer containing heptakis(2,6-di-O-methyl)-β-cyclodextrin [8], it was concluded that the ephedrines present in these supplements were derived from natural sources. The separation efficiency that is inherent to CE also facilitates the separation of amphetamines, while allowing the simultaneous visualization of their potential precursors.

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