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Enantiomeric separation of atropine in *Scopolia* extract and *Scopolia Rhizome* by capillary electrophoresis using cyclodextrins as chiral selectors

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Abstract

We separated the enantiomers of atropine, a main ingredient of *Scopolia* extract and *Scopolia Rhizome*, by capillary electrophoresis. The best conditions for chiral separation were investigated based on the concentration and type of cyclodextrin (CD) used, the pH, the concentration of the electrolyte solution and the capillary temperature. Good resolution of D- and L-hyoscyamine (atropine) was achieved in 100 m*M* phosphate buffer (pH 2.5) containing 30 m*M* trimethyl-β-cyclodextrin (TM-β-CD) as the chiral selector. The calibration curves showed good linearity in the range of 10–200 μg/ml ($r \geq 0.99$) for D-hyoscyamine, L-hyoscyamine and scopolamine. We could analyze atropine from the samples of crude drugs and pharmaceutical preparations according to the procedures described in The Japanese Pharmacopoeia (Thirteenth Edition).

Keywords: Enantiomer separation; Pharmaceutical analysis; Atropine; Cyclodextrins; Hyoscyamine; Scopolamine

1. Introduction

Gas chromatography (GC) [1–3] and high-performance liquid chromatography (HPLC) [3–5] have played a major role in the chiral separation of pharmaceutical and natural products. However, GC is limited to volatile compounds, and HPLC sometimes shows poor separation efficiencies, resulting in a lack of baseline resolution and the need for expensive chiral columns. For the last few years, CE has become a useful tool in the separation of enantiomers, using chiral selectors. In addition, CE can achieve rapid and high resolution separation of water-soluble compounds present in a small sample volume.

The common chiral selectors in CE are divided into three categories; inclusion systems (e.g., cyclodextrins or crown ethers), optically active surfactants

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(chiral mixed micelle systems or bile salts) and enantioselective metal complexation. Among them, cyclodextrins have been widely used for the separation of enantiomers. Cyclodextrins, of which the hydroxyl groups are substituted by either methyl or hydroxypropyl ethers at positions 2, 3 and 6, are also employed, because these derivatives afford enhanced solubility and enantioselectivity.

Scopolia extract and *Scopolia Rhizome* are crude drugs made from the root of *Scopolia japonica*, which belongs to the *Solanaceous* plants. *Scopolia* extract contains tropane alkaloids, such as atropine and scopolamine, as the main ingredients. The content is 0.9–1.0% of the total amount of alkaloids. Atropine is also called DL-hyoscyamine, racemized during extraction, and consists of a mixture of equal parts of D- and L-hyoscyamine [6]. The tropane alkaloids have parasympathicus block action and are widely used as anti-cholinergic drugs. The antimuscarinic activity is, however, almost totally due to the naturally occurring L-form [7]. L-Hyoscyamine is twice as potent as the atropine racemate in its antimuscarinic activity [8].

The enantiomeric separation of atropine has been reported using thin-layer chromatography [9] and HPLC [10,11]. In CE, a few attempts to separate the enantiomers of atropine were unsuccessful [12–14]. Rawjee et al. [15] described the separation of atropine using a neutral capillary and hydroxypropyl-β-cyclodextrin (HP-β-CD) as the chiral selector, but the analysis required approximately 40 min. Tanaka and Terabe [16] also reported the separation of atropine using a carrier containing α_1-acid glycoprotein (α_1-AGP) as a chiral selector and 10% 2-propanol as an organic modifier. Separations of atropine using HP-α-CD and TM-β-CD were also reported, but the resolution between D-hyoscyamine and L-hyoscyamine was not optimized [17,18]. Recently, Nassar et al. [19] succeeded in the chiral separation of atropine using a negatively charged cyclodextrin, sulfobutylether-β-CD, as the chiral selector.

In the present study, the chiral separation of atropine was extensively investigated using CDs for the analysis of a mixture of tropane alkaloids in an extract of crude drugs and pharmaceutical preparations.

2. Experimental

2.1. Materials

The atropine, L-hyoscyamine and scopolamine hydrobromide were obtained from Tokyo Kasei (Tokyo, Japan). The α-, β- and γ-cyclodextrins were purchased from Wako (Osaka, Japan). TM-β-CD, DM-β-CD, M-β-CD and 2-HP-β-CD were obtained from Sigma (St. Louis, MO, USA). According to the product sheet, at least 90% of the hydroxy groups of TM-β-CDs were substituted with methyl groups. All other reagents used in this work were of analytical reagent grade, or HPLC grade.

2.2. Apparatus

All CE experiments were performed with a P/ACE 5510 system (Beckman, Fullerton, CA, USA) equipped with a photodiode array detector, an autosampler and a temperature-controller (15–40±0.1°C). The instrument was equipped with a data-handling system comprising a Compaq personal computer and 'P/ACE station' software.

2.3. Electrophoretic procedure

The separation was preformed in a fused-silica capillary (50 μm I.D.; effective length, 40 cm; total length, 47 cm; Beckman). Detection was performed by monitoring the absorbance at 214 nm. The system was operated at 15 kV in a constant-voltage mode. The observed current varied between 20 and 100 μA at 15 kV, depending on the buffer concentration and pH. The capillary temperature was examined in the range of 15–50°C. The sample was injected in pressure mode at 3.45 kPa for 2 s. The running buffer was prepared by mixing 100 m*M* phosphoric acid and 100 m*M* sodium hydroxide, using a pH meter (TOA HM-26S) to monitor the pH, and was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA). The capillary was flushed with 0.1 *M* sodium hydroxide for 10 min, followed by distilled water for 5 min, prior to analysis. In order to achieve high reproducibility of migration times and to avoid adsorption of the solute, the capillary was washed between runs with 0.1 *M*

sodium hydroxide for 2 min, followed by water for 1 min, then equilibrated with the running buffer for 5 min.

2.4. Calculation of resolution

The resolution (R) of the enantiomers was calculated using the equation

$$R = \frac{2(T_2 - T_1)}{W_2 + W_1}$$

where T_1 and T_2 (s) are the migration times, and W_1 and W_2 (s) are the widths of the peaks at the baseline [20].

2.5. Analysis of the Scopolia extract and Scopolia Rhizome

The *Scopolia* extract and *Scopolia Rhizome* were preprocessed according to The Japanese Pharmacopoeia (Thirteenth Edition) (JP13), as follows [21]. A 0.4-g amount of a sample was weighed accurately in a centrifuge tube made from poly(tetrafluoroethylene). A 10% ammonia solution (15 ml) and 25 ml of ether were added. After shaking the mixture for 10 min, the organic phase was separated from the aqueous phase by centrifugation at 2500 *g* for 10 min. The organic phase was evaporated to dryness in a water bath at 40°C. The residue was dissolved in 10 ml of distilled water and filtered through a 0.22-μm membrane filter (Millipore), and a portion of the filtrate was analyzed by CE.

3. Results and discussion

3.1. Selection of CD

For the chiral separation of atropine enantiomers, it is important to select the proper CD. The effect of the CD in the running buffer was investigated using 100 m*M* phosphate buffer (pH 2.5) containing each of the CDs (α-, β-, γ-, M-β-, DM-β-, TM-β- and 2-HP-β-CD). The results are summarized in Table 1. The concentration of the CDs was adjusted to 30 m*M* for each CD, except for β-CD, which was adjusted to 15 m*M*. Chiral separations of the atropine were observed when using γ-CD, M-β-CD and TM-β-CD as the additive. The separation profiles are shown in Fig. 1, and TM-β-CD gave the best separation.

Higher concentrations of TM-β-CD gave better resolution, as shown in Fig. 2. To achieve complete resolution ($R = 1.5$) of hyoscyamine enantiomers, concentrations of TM-β-CD higher than 30 m*M* were required. The migration times of D- and L-hyoscyamine were 15.7 and 15.9 min, respectively, using 30 m*M* TM-β-CD. Hence, we employed this concentration for the analysis of pharmaceutical preparations.

3.2. Optimization of the analytical conditions

The buffer concentration and pH should also be considered during the chiral separation of atropine enantiomers. In particular, the pH of the electrolyte significantly affects the velocity of the electroosmotic flow (EOF) in free-zone electrophoresis. Low pH

Table 1
Resolution and migration time of D,L-hyoscyamine

Cyclodextrin	Concentration used (m*M*)	Migration time (min)		Resolution
		L-HYO[a]	D-HYO[b]	
α-Cyclodextrin (α-CD)	30	16.6	–	0.00
β-Cyclodextrin (β-CD)	15	20.8	–	0.00
γ-Cyclodextrin (γ-CD)	30	21.4	21.6	0.55
Methyl-β-cyclodextrin (M-β-CD)	30	30.9	31.1	0.55
Dimethyl-β-cyclodextrin (DM-β-CD)	30	36.5	–	0.00
Trimethyl-β-cyclodextrin (TM-β-CD)	30	15.7	15.9	1.53
Hydroxypropyl-β-cyclodextrin (HP-β-CD)	30	33.0	–	0.00

[a] L-HYO, L-hyoscyamine.
[b] D-HYO, D-hyoscyamine.

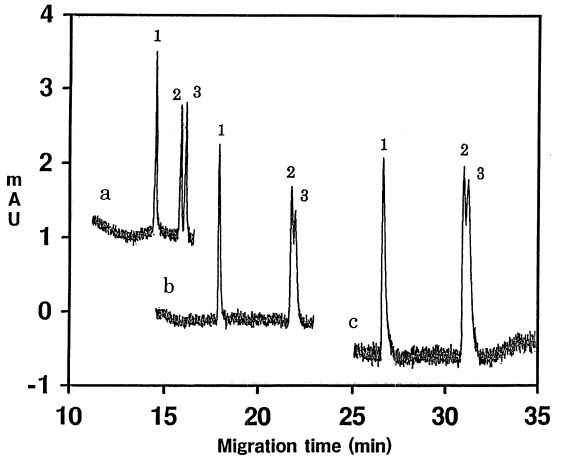

Fig. 1. Electropherograms of enantiomeric separation using CE with several CDs. (a) TM-β-CD, (b) γ-CD and (c) M-β-CD. Conditions: electrolyte, 100 m*M* phosphate (pH 2.5) containing 30 m*M* each CD; analyte concentration, 100 μg/ml; applied voltage, 15 kV; temperature, 20°C; capillary, fused-silica capillary, 47 cm×50 μm I.D.; UV detection was at 214 nm. 1, scopolamine; 2, L-hyoscyamine and 3, D-hyoscyamine

leads to lowering of the EOF and an increase in the migration time window and, therefore, may improve the resolution and enantioselectivity during chiral separation. The effect of buffer pH was investigated using 30 m*M* TM-β-CD and 100 m*M* phosphate buffer over the pH range of 2.0–7.5 (Fig. 3a). By using an acidic buffer at around pH 2.5, electroendosmosis is small and the duration of the interaction between TM-β-CD and atropine becomes large, enhancing the resolution between the enantiomers.

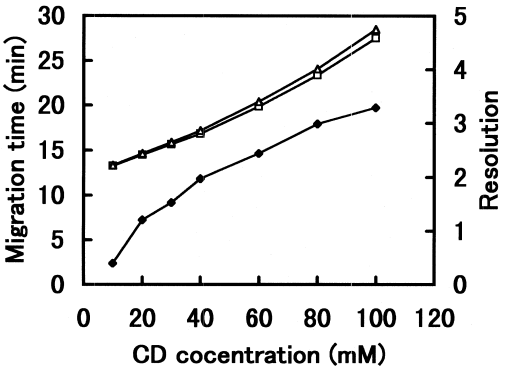

Fig. 2. Migration time and resolution of atropine as a function of TM-β-CD concentration (20–100 m*M*). For other conditions, see Fig. 1. □=Migration time of L-hyoscyamine, △=migration time of D-hyoscyamine and ♦=resolution of atropine.

Fig. 3b shows the effect of the concentration of phosphate buffer on chiral separation. Concentrations higher than 100 m*M* phosphate buffer had almost the same resolution, therefore, 100 m*M* phosphate buffer was chosen for further investigation.

The temperature used is also important in CE [22–24]. The electrophoretic experiments were performed in the temperature range of 15 to 50°C using 100 m*M* phosphate buffer, pH 2.5, containing 30 m*M* TM-β-CD in order to study the effect of capillary temperature on the enantiomeric resolution and migration time, as shown in Fig. 4. Increasing the capillary temperature caused a decrease in migration times and resolution because the rise in temperature caused a decrease in the viscosity of the running electrolyte, thus increasing both the EOF and electrophoretic mobilities. The secondary effect might be a decrease in the equilibrium constant in inclusion of enantiomers into CD molecules because the interaction between the solute molecule and CD becomes rapid at higher temperatures. However, temperatures below 20°C were not accurate due to difficulty in controlling room temperature in our laboratory. Therefore, the operation temperature was set at 20°C for further investigation.

3.3. Linearity and limits of detection

The calibration curves for tropane alkaloids showed good linearity in the range of 10–200 μg/ml ($r \geq 0.99$) for D-hyoscyamine, L-hyoscyamine and scopolamine. The limit of detection was determined to be 1 μg/ml at $S/N=3$. The reproducibilities (R.S.D., $n=5$), on the basis of migration time and peak area, respectively, were 0.88 and 5.10% for scopolamine, 0.94 and 1.03% for L-hyoscyamine and 0.96 and 6.35% for D-hyoscyamine. We examined linearity using a mixture of scopolamine, D- and L-hyoscyamine. Reproducibility in the determination of D-hyoscyamine was worse than that for L-hyoscyamine due to the preceding L-hyoscyamine peak. However, good results were obtained in accuracy studies on both migration times and quantitation, even without the use of an internal standard.

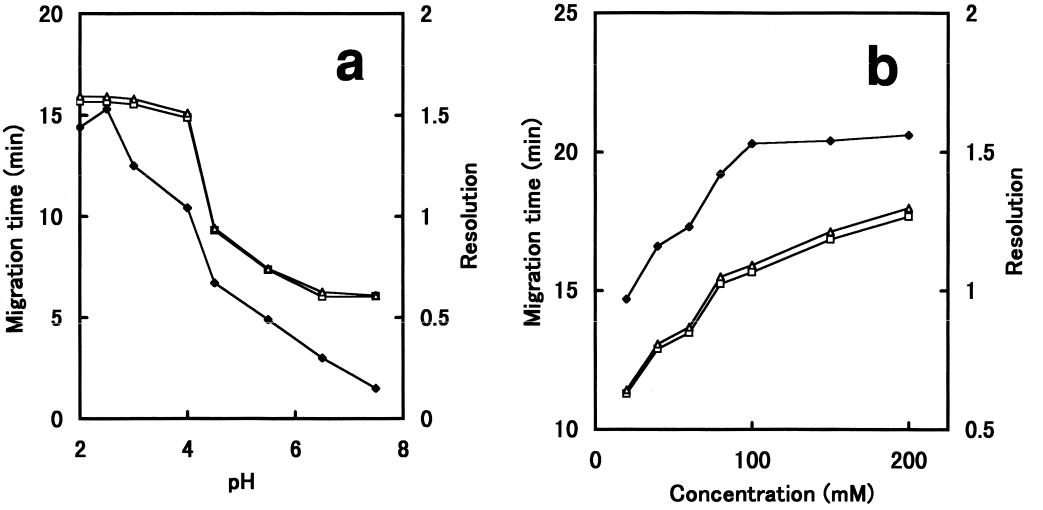

Fig. 3. Migration time and resolution of atropine as a function of (a) pH and (b) buffer concentration. For other conditions, see Fig. 1. □=Migration time of L-hyoscyamine, △=migration time of D-hyoscyamine and ♦=resolution of atropine.

3.4. Assay of crude drugs and pharmaceutical preparations

Tropane alkaloids in the crude drugs and pharmaceutical preparations were analyzed by several methods using HPLC [25–27] and CE [28]. To our knowledge, there has been no report on the chiral separation of atropine in pharmaceutical preparations by CE.

A solution of the mixture of scopolamine hydrobromide and atropine was separated under the optimized conditions, and the electropherogram is shown in Fig. 5 a. Fig. 5(b) and (c) shows the typical electropherograms of the samples of *Scopolia* extract and *Scopolia Rhizome*, from which atropine was extracted according to the JP13, as described in

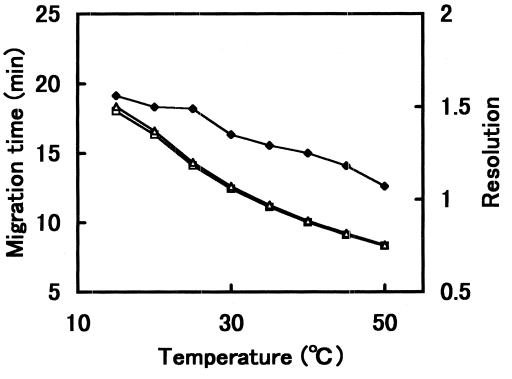

Fig. 4. Migration time and resolution of atropine as a function of capillary temperature (15–50°C). For other conditions, see Fig. 1. □=Migration time of L-hyoscyamine, △=migration time of D-hyoscyamine and ♦=resolution of atropine.

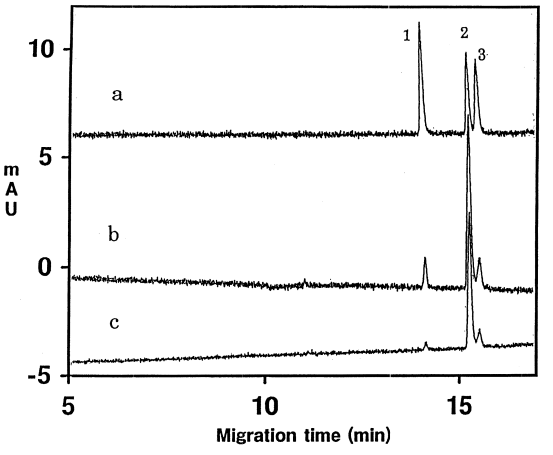

Fig. 5. Electropherograms of (a) a mixture of atropine and scopolamine hydrobromide (100 μg/ml water), (b) *Scopolia* extract and (c) *Scopolia Rhizome*. 1, scopolamine; 2, L-hyoscyamine and 3, D-hyoscyamine.

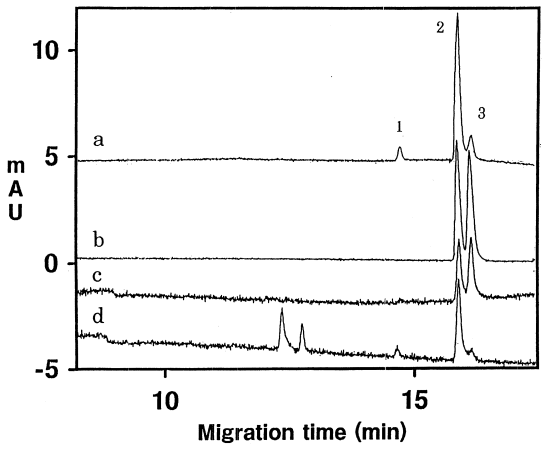

Fig. 6. Electropherograms of (a) *Scopolia* extract, (b) atropine sulfate injection, (c) eye drop and (d) gastrointestinal drug (powder). 1, scopolamine, 2, L-hyoscyamine and 3, D-hyoscyamine

Section 2. The electropherograms are generally simple, and no interference peak was observed.

In relation to the application, we determined the contents of tropane alkaloids in various pharmaceutical preparations. The electropherograms of some preparations are shown in Fig. 6, and the results are summarized in Table 2. All preparations in Fig. 6 clearly indicate that they contained different amounts of D- and L-hyoscyamine. Several research groups have reported the presence of littorine, a precursor of hyoscyamine in *Datura stramonium* [29]. In the present study, we could not determine littorine in pharmaceutical preparations, because the standard sample of littorine was not commercially available. In Fig. 6(d), two large peaks were observed at around 12.5 min. This preparation was composed of different ingredients. These peaks were probably due to these ingredients. The average recoveries ($n=5$) obtained for the analysis of D- and L-hyoscyamine spiked in a *Scopolia* extract powder were 83.4 and 82.9%, respectively. Interestingly, there were many variations in the amount and the ratio of D- and L-hyoscyamine. Both the atropine injection and eye drop preparations contained atropine, and the ratio of D- and L-hyoscyamine was almost unity. However, other pharmaceutical preparations that contained *Scopolia* extract had various amounts of D- and L-hyoscyamine. These differences were probably due to the processing procedures of the original plants. These variations may cause differences in biological activities. The present technique will be useful for such an evaluation.

4. Conclusions

Atropine was enantiomerically separated in 100 m*M* phosphate buffer (pH 2.5) containing 30 m*M* TM-β-CD. Baseline resolution between D- and L-hyoscyamine was achieved under these conditions. According to the protocol described in the JP13, we

Table 2
Determination of atropine in pharmaceutical preparations

Pharmaceutical preparation	Content of atropine[a] or *Scopolia* extract[b] (%)	Analysis method	Content of	
			L-HYO[c]	D-HYO[c]
Scopolia extract powder	10[b]	1[c]	6.9±1.0 mg/g (83.3)	1.4±6.6 mg/g (16.7)
Atropine sulfate injection	0.05[a]	2[c]	0.2±1.8 mg/ml (50.2)	0.2±2.3 mg/ml (49.8)
Eye drop	1[a]	2	5.2±0.9 mg/ml (51.1)	5.0±2.3 mg/ml (48.9)
Gastrointestinal drug A (tablet)	0.64[b]	1[c]	16.0±1.0 μg/g (80.6)	3.8±5.3 μg/g (19.4)
Gastrointestinal drug B (tablet)	0.84[b]	1[c]	18.5±0.9 μg/g (90.3)	2.0±5.3 μg/g (9.7)
Gastrointestinal drug C (tablet)	0.67[b]	1[c]	21.7±0.4 μg/g (90.1)	2.4±4.8 μg/g (9.9)
Gastrointestinal drug D (powder)	0.66[b]	1[c]	33.2±0.4 μg/g (84.9)	5.9±2.9 μg/g (15.1)
Gastrointestinal drug E (powder)	0.69[b]	1[c]	29.9±0.4 μg/g (81.7)	6.7±4.0 μg/g (18.3)
Gastrointestinal drug F (powder)	0.64[b]	1[c]	28.6±1.0 μg/g (73.8)	10.1±2.3 μg/g (26.2)

[a] The preparations contain atropine.
[b] The preparations contain *Scopolia* extract.
[c] L-HYO: L-hyoscyamine, D-HYO: D-hyoscyamine. Method 1: prepared according to the procedure described in Section 2. Method 2: Diluted with distilled water. Values in parentheses are expressed in weight percent of the total D- and L-hyoscyamine.

could achieve accurate determination of scopolamine and DL-hyoscyamine in pharmaceutical preparations.

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