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Development and validation of a capillary zone electrophoresis method for the determination of atropine, homatropine and scopolamine in ophthalmic solutions

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Abstract

A capillary zone electrophoresis method is described for the simultaneous determination of atropine, homatropine and scopolamine. Successful results were obtained after optimization of the electrophoretic parameters such as buffer composition and pH. The best separation was achieved using a 100 m*M* Tris–phosphate running buffer at pH 7. The validation data proved that the method had the requisite selectivity, sensitivity, reproducibility and linearity to be used for the assay of these compounds in pharmaceutical formulations. Dosage of the separate drugs in ophthalmic preparations is also presented. © 1997 Elsevier Science B.V.

Keywords: Atropine; Homatropine; Scopolamine; Tropane alkaloids

1. Introduction

Atropine, homatropine and scopolamine belong to the alkaloid family and have various pharmaceutical applications. They are extensively used in ophthalmic diagnosis as mydriatic. They are also used as anticholinergic, antispasmodic and preanesthesic agents. Atropine and scopolamine, also referred to as (±)-hyoscyamine and hyoscine, respectively, are extracted from plant species belonging to the *Solanaceae* family. Homatropine, prepared synthetically by esterification of mandelic acid with 3α-tropine, is structurally related to atropine and scopolamine, as seen in Fig. 1.

During recent years, thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been widely used for the analysis and the quantitation of tropane alkaloids. However, due to the low volatility and thermosensitivity of these compounds, GC has so far found only limited applications [1]. HPLC analysis frequently requires the addition of a basic component as a masking agent (e.g., triethylamine), the use of shielded phases, or the addition of a pairing reagent in the mobile phase in order to reduce the peak tailing and to improve the column efficiency and peak shapes [2–4].

Capillary zone electrophoresis (CZE) represents an interesting alternative for the analysis of pharmaceutical compounds because of its efficiency, flexibility, accuracy and very high resolution [5]. Capillary electrophoresis (CE) has been successfully applied to the analysis of pharmaceutical products [6–10], but the first review on the application of CE

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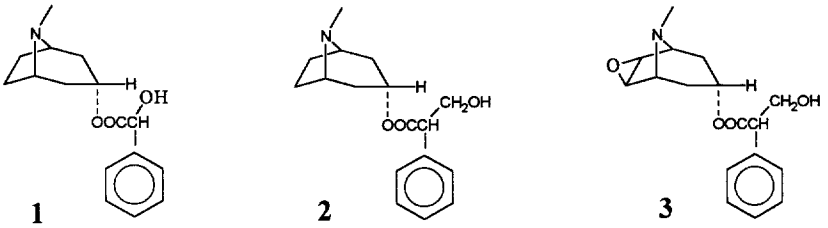

Homatropine
MW=275

Atropine
MW=289
pKa = 9.85 (21°C)

Scopolamine
MW=303
pKa = 7.6 (23°C)

Fig. 1. Structure and MW of the studied tropane alkaloids.

for the analysis of plant secondary metabolites has only been published recently [11]. Phenolic compounds and flavonoids in particular have been widely investigated by CE [12–15], but surprisingly there have been only a couple of reports concerning the analysis of tropane alkaloids [16,17].

In the course of our continuing investigations on the development of straightforward approaches to the analysis of drugs of pharmaceutical interest, we report here on the development and the validation of a CZE method for the simultaneous determination of atropine, homatropine and scopolamine (see Fig. 1). The validated method is applied to the quantitation of these compounds in commercially available ophthalmic solutions.

2. Experimental

2.1. Materials

Atropine sulfate, scopolamine hydrobromide and homatropine hydrobromide were supplied by Sigma (St. Louis, MO, USA). Atropine (1%, w/v) and scopolamine (0.25%, w/v) ophthalmic solutions were supplied by Ciba Vision (Niederwangen, Switzerland). Isopto-Homatropine (1%, w/v) was purchased from Alcon (Rueil Malmaison, France). Atropine is present as the sulfate form, while homatropine and scopolamine are in the hydrobromide form.

All chemicals, including tris(hydroxymethyl)-aminomethane (Tris), sodium dihydrogen phosphate and sodium hydroxide were of analytical grade and were purchased from Fluka (Buchs, Switzerland). Doubly-distilled water was used for standard and sample preparation.

2.2. Instrumentation and electrophoretic procedure

Electrophoresis was carried out on a HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode-array detector (DAD). The capillary (Hewlett-Packard) was 64.5 cm (56 cm effective length)×50 μm I.D.. An alignment interface containing optical slit matched to the internal diameter was used. Detection at 56 cm from the point of sample introduction was set at 195 nm with a bandwidth of 10 nm. A CE Chemstation (Hewlett-Packard) was used for instrument control, data acquisition and data handling.

All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). The capillary was thermostated at 25°C. A constant voltage of 30 kV, with an initial ramping of 500 V s^{-1}, was applied during analysis. Sample injections (12 nl injection volume) were achieved using the pressure mode for 20 s at 25 mbar.

Each day, silica capillaries were flushed with 1 *M* sodium hydroxide for 15 min, followed by water for 10 min. To achieve high migration time reproducibility and to avoid solute adsorption, the capillary was washed between analyses with 0.1 *M* sodium hydroxide for 2 min, followed by water for 2 min, then equilibrated with the running buffer for 3.5 min.

As electrolysis of the buffer solution can alter the running buffer and subsequently change the electroosmotic flow (EOF), a replenishment system was also used to maintain a high reproducibility. Prior to each sequence, two blank injections were performed

to stabilize the capillary wall surface, and allowing the buffer and sample solutions to reach a constant temperature on the autosampler tray. Triplicate injections of the solutions were performed. The resolution between two consecutive peaks was calculated using the equation: $R_s=2[(t_2-t_1)/(w_2+w_1)]$, where t_2 and t_1 are the migration times and w_2 and w_1 are the base peak widths of solutes 2 and 1, respectively.

A mixture of Tris and sodium dihydrogen phosphate solutions was used to prepare buffers in order to cover a pH range between 6 and 8 in the optimization experiments. All sample and buffer solutions were filtered through 0.20 μm filter (Supelco, Bellefonte, USA) before use.

2.3. Standard and ophthalmic solutions

2.3.1. Standard solutions

Stock standard solutions were prepared by dissolution of each compound in methanol (1 mg ml^{-1}) and then suitably diluted in water to obtain standard solutions at a final concentration from 0.01 to 0.125 mg ml^{-1}. The use of water as dissolving agent allowed sample stacking which was effective in enhancing sensitivity by on-column preconcentration within the capillary.

2.3.2. Ophthalmic solutions

Each ophthalmic solution was composed of the alkaloid, benzalkonium chloride as preservative, methylhydroxypropylcellulose as ophthalmic lubricant and sodium chloride as isotonic agent. The analysis was carried out without an extraction process; the aqueous solution was only diluted with doubly-distilled water to the desired concentration (ca. 0.05 mg ml^{-1}).

3. Results and discussion

3.1. Method development

In our first attempts to set up a CZE method for the analysis of the selected tropane alkaloids, acidic pH was chosen because, under these conditions, all three selected compounds are positively charged, hence migrating as cations towards the cathode. 50 m*M* Tris buffer titrated to pH 2.5 with concentrated phosphoric acid was selected for this purpose. At this pH, as mentioned above, the three selected alkaloids are positively charged and their migration is mainly controlled by their electrophoretic mobility. However, because of their structural similarity, the resolution of the three alkaloids was not sufficient to pursue our investigations in this pH region. The effect of pH (between 2.5 and 3.5) as well as addition of organic modifier such as acetonitrile and methanol was also investigated. No improved resolution was observed.

With regard to these results obtained at acidic pH, we decided to work around the p*K* of scopolamine (7.6) to ensure changes in the degree of ionization, and subsequently to improve compound resolution. Thus, Tris buffer titrated with sodium dihydrogen phosphate, in the pH range from 6 to 8, was then selected to investigate the effect of the concentration and the pH on resolution.

3.2. Buffer concentration

The influence of buffer concentration on compound resolution was investigated at pH 7. Among the capillaries tested for buffer concentration studies, 50 and 75 μm I.D., only the former permits the use of high ionic strengths owing to better heat dissipation. The plot of resolution versus buffer concentration is reported in Fig. 2. In CZE, the migration behavior of each compound is the result of the electrophoretic mobility of the analyte and the electroosmotic mobility. As the electrophoretic mobility is directly proportional to the compound's total charge, scopolamine, which is partially protonated at pH 7, is less influenced by buffer concentration increase, which explains the observed decrease in resolution between scopolamine and atropine. The resolution between homatropine and atropine remains almost constant as both electrophoretic mobilities are modified in the same magnitude by the concentration changes. In addition, working at high buffer concentration allows improved sensitivity as a result of sample stacking during injection. However, high buffer concentration will induce excessive heating caused by Joule effect, resulting in an unstable method. Consequently, a 100

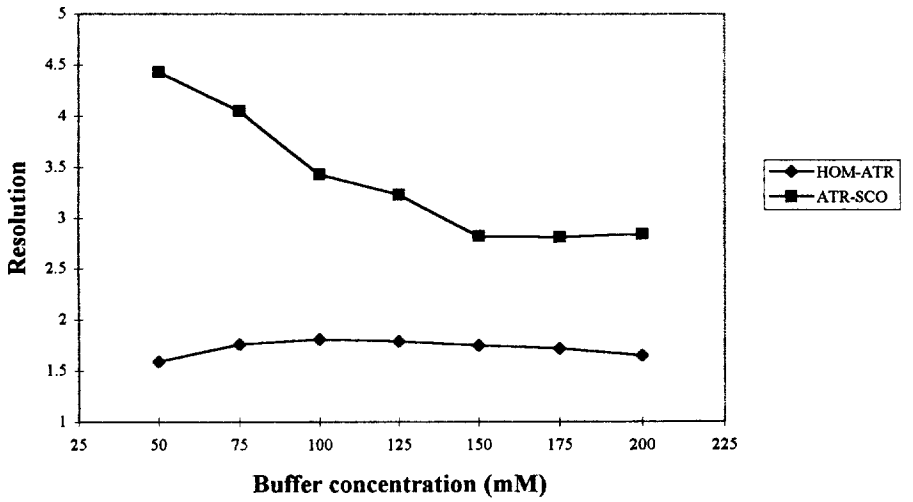

Fig. 2. Influence of Tris–phosphate concentration on homatropine/atropine and atropine/scopolamine resolution. Conditions: buffer pH 7, applied voltage: 30 kV, temperature 25°C. Other conditions are given in Section 2.2.

m*M* Tris–phosphate buffer was selected for further analyses.

3.2.1. Buffer pH

The buffer pH is a crucial parameter to improve resolution of closely related compounds. As already mentioned above, separation at a pH value closer to the pK_a of scopolamine (which is the lowest one) is expected to achieve better resolution. The effect of pH was investigated in a pH range between 6 and 8, at a fixed buffer concentration (100 m*M*).

Scopolamine, which is partially protonated in this pH range elutes after homatropine and atropine but before the EOF. As shown in Fig. 3, the resolution between atropine and scopolamine increases dramatically as a function of the pH, because the atropine

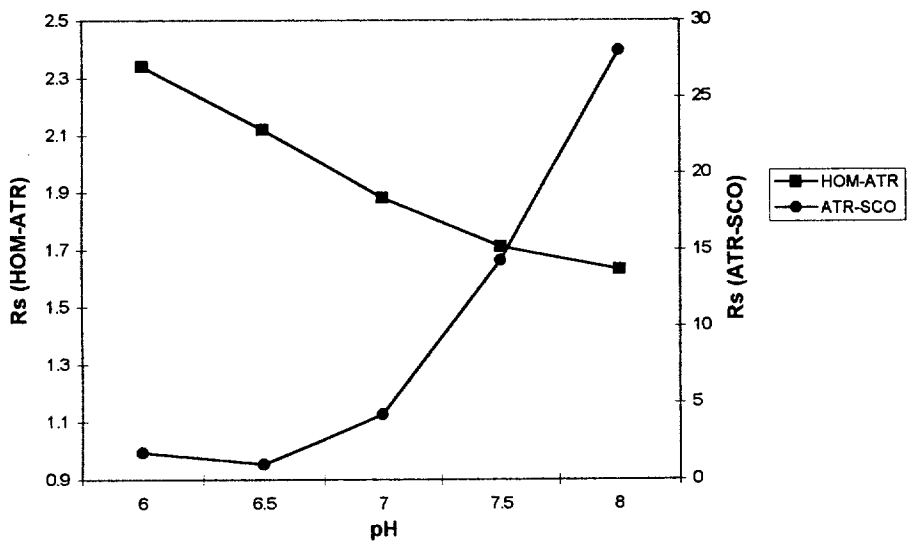

Fig. 3. Influence of Tris–phosphate pH on homatropine/atropine and atropine/scopolamine resolution. Conditions: Tris–phosphate concentration: 100 m*M*, applied voltage: 30 kV, temperature 25°C. Other conditions are given in Section 2.2.

charge remains constant from pH 6 to 8, while scopolamine ionicity is modified in this pH range. Indeed, pH increase from 6.5 to 8 results in resolution increase from 2 to 28. However, under the same conditions, the resolution between atropine and homatropine, which are cationic species, decreases slightly as a function of the pH, owing to the EOF increase.

Using a 50 μm uncoated fused-silica capillary, 100 m*M* Tris–phosphate buffer at pH 7 yielded the best compromise in terms of analysis time, selectivity and separation efficiency. This buffer was used in subsequent stages of method validation. Fig. 4 shows a typical electropherogram obtained under the selected conditions; all three compounds were separated within 4 min.

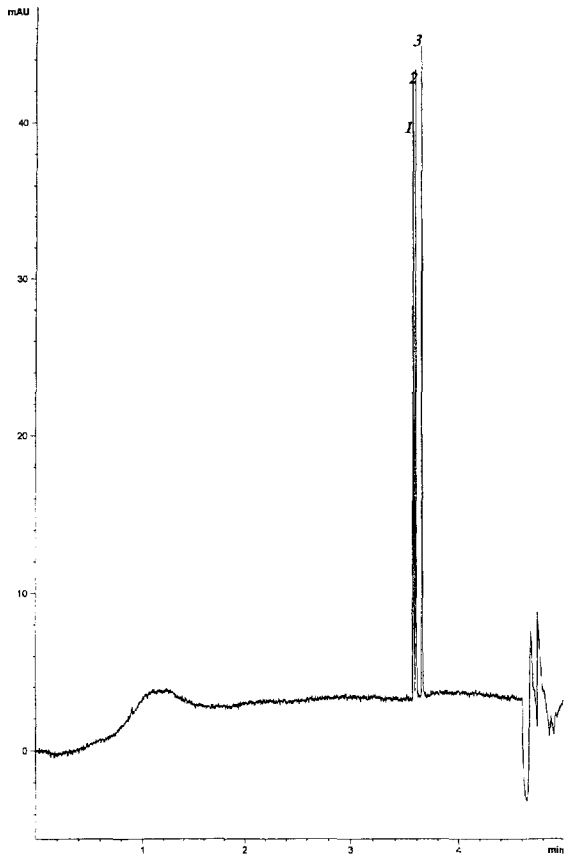

Fig. 4. Typical electropherogram of homatropine, atropine and scopolamine obtained by CZE using 100 m*M* Tris–phosphate buffer at pH 7. Other operating conditions: capillary: uncoated fused-silica, L=64.5 cm, l=56 cm, I.D.=50 μm; applied voltage: 30 kV (i=63 μA), temperature 25°C. Other conditions are given in Section 2.2..

3.3. Method validation

The optimized method was validated for atropine, homatropine and scopolamine quantitation. The validation was carried out in a similar way to that generally adopted for HPLC and now employed to validate CE methods [18]. The procedure requires the assessment of migration time and peak area reproducibility, detector response linearity with sample concentration, sensitivity and recovery.

3.3.1. Precision

In order to determine the repeatability (within-day precision) of the method, replicate injections (n=6) of 0.05 mg ml^{-1} mixture solution containing homatropine, atropine and scopolamine were carried out. In Table 1, the average values and relative standard deviations (R.S.D.s) are given for the migration time and peak area. In all cases, the precision was better than 0.6% for the migration time and 3.2% for the peak area. Good peak area precision was achieved

Table 1
Precision of CE migration time and peak area for replicates injections (n=6) of homatropine, atropine and scopolamine standard solutions prepared at 0.05 mg ml^{-1}

	Migration time (min)		Area	
	Average	R.S.D.(%)	Average	R.S.D.(%)
Day 1				
Homatropine	3.448	0.08	20.775	3.19
Atropine	3.469	0.07	20.921	2.69
Scopolamine	3.531	0.46	22.386	3.00
Day 2				
Homatropine	3.676	0.52	21.204	2.93
Atropine	3.701	0.52	21.377	2.47
Scopolamine	3.765	0.53	22.889	2.28
Day 3				
Homatropine	3.567	0.55	21.300	1.70
Atropine	3.591	0.56	21.622	1.17
Scopolamine	3.636	0.57	23.360	1.34
Average				
Homatropine	3.564	0.38	21.093	2.61
Atropine	3.587	0.38	21.307	2.11
Scopolamine	3.644	0.52	22.879	2.21

without adding any internal standard to circumvent injection variation or peak area normalization (dividing the main peak area by its corresponding migration time).

The reproducibility (between-day precision) was also evaluated over 3 days by performing six successive injections each day. The results (Table 1) show that the reproducibility of migration time and peak area were satisfactory. The reproducibility (R.S.D.), on the basis of migration time and peak area were respectively, 0.38% and 2.61% for homatropine, 0.38% and 2.11% for atropine and 0.52% and 2.21% for scopolamine.

3.3.2. Linearity

The linearity of the method (peak area versus concentration) was evaluated over a concentration range from 0.01 to 0.125 mg ml^{-1} for atropine, homatropine and scopolamine. Good linearity in terms of peak area response as a function of analyte concentration is demonstrated by the high correlation coefficients ($r>0.99$) observed for the regression lines. The regression analysis data, calculated by the least squares method, for atropine, homatropine and scopolamine are shown in Table 2. The intercept values were not significantly different from zero (Student t-test).

3.3.3. Limits of detection (LODs) and quantification (LOQs)

The limit of detection, defined as the lowest concentration of analyte that can be clearly detected above the baseline signal, is estimated as three times the signal-to-noise ratio. The LOD was determined by injecting test mixture solutions of various concentrations. The estimated limit of detection (Table 2) was determined as 1 μg ml^{-1} giving a limit of quantitation (LOQ) value of 3 μg ml^{-1} for each drug examined.

3.4. Drug determination in ophthalmic solutions

The determination of the alkaloids was performed on commercially available aqueous ophthalmic solutions with the method described above. It has to be noted that atropine, homatropine and scopolamine are not present together in the different formulations tested. Fig. 5 shows typical electropherograms for the determination of each alkaloid in commercial ophthalmic solutions. In all instances, the selected alkaloids could be easily determined without any sample pretreatment and no disturbance was observed due to the presence of excipient and preservative in these ophthalmic solutions.

As can be seen from Table 3, determination of drug content by the proposed method was in good agreement with the labeled content. The R.S.D. values attest to the precision of the method even if the use of internal standard will clearly result in precision improvement both for migration time and peak area response. These results suggest that this method can be successfully applied for the routine determination of tropane alkaloids in pharmaceutical formulations.

4. Conclusions

CZE was investigated for the separation of three tropane alkaloids. Once the appropriate buffer system was selected, the pH and concentration were optimized so as to obtain the best resolution. CZE performed in a 50 μm uncoated fused-silica capillary, in the presence of 100 mM Tris–phosphate buffer at pH 7, offers a suitable and rapid method for

Table 2
Regression data and LOD for atropine, homatropine and scopolamine

	Regression data				
	Range (mg ml^{-1})	Line	r	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
Atropine	0.01–0.125	$y=497.92x-2.339$	0.9970	1	3
Homatropine	0.01–0.125	$y=497.78x-2.7091$	0.9972	1	3
Scopolamine	0.01–0.125	$y=531x-2.3462$	0.9968	1	3

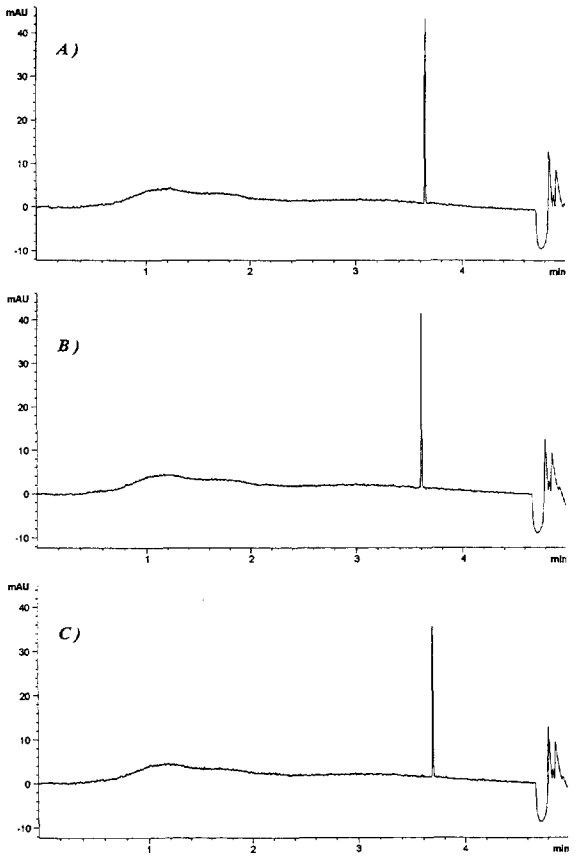

Fig. 5. Typical electropherograms of tropane alkaloid determination in ophthalmic solutions. (A) Atropine, (B) homatropine and (C) scopolamine. Other operating conditions are the same as for Fig. 4.

the simultaneous determination of atropine, homatropine and scopolamine.

This method has been validated and shows a good performance with respect to selectivity, reproducibility, linearity and accuracy. Owing to its simplicity and rapidity, the validated method can be a good tool for the assay of tropane alkaloids in dosage forms such as ophthalmic solutions. Works are in progress to apply CE to the determination of tropane alkaloids in plant extracts.

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Table 3
Results of CZE assay of atropine, homatropine and scopolamine in ophthalmic solutions ($n=3$)

	Dosage formulation		
	Atropine 1% Dispersa Ciba Vision	Isopto-homatropine 1% Alcon	Scopolamine 0.25% Ciba Vision
Component	*Atropine*	*Homatropine*	*Scopolamine*
Labeled claim (%, w/v)	1	1	0.25
Amount found (%, w/v)	1.04	0.98	0.24
R.S.D. (%) (peak area)	1.82	3.50	2.31
R.S.D. (%) (migration time)	0.18	0.02	0.07

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