Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 623–633
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Impurity profiling of atropine sulfate by microemulsion electrokinetic chromatography

Yaser Bitar, Ulrike Holzgrabe *

Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

Received 20 June 2006; received in revised form 20 July 2006; accepted 21 July 2006
Available online 12 September 2006

Abstract

An oil-in-water microemulsion electrokinetic chromatography (MEEKC) method has been developed and validated for the determination of atropine, its major degradation products (tropic acid, apoatropine and atropic acid) and related substances from plants material (noratropine, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine and littorine).

Separation of atropine and all impurities was optimized by varying the voltage, the nature of the oil droplet and the buffer, as well as the organic modifier (methanol, 2-propanol or acetonitrile) and the surfactant type and concentration. The optimum O/W microemulsion background electrolyte (BGE) solution consists of 0.8% (w/w) octane, 6.62% (w/w) 1-butanol, 2.0% (w/w) 2-propanol, 4.44% (w/w) SDS and 86.14% (w/w) 10 mM sodium tetraborate buffer pH 9.2. In order to shorten the analysis time a voltage gradient was applied. The validation was performed with respect to specificity, linearity, range, limit of quantification and detection, precision, accuracy and robustness. The established method allowed the detection and determination of atropine sulfate related substances at impurity levels given in the *European Pharmacopoeia*. Good agreement was obtained between the established MEEKC method and the traditional RP-HPLC method.

Keywords: Atropine; Tropa alkaloids; Microemulsion electrokinetic chromatography; Impurity profiling

1. Introduction

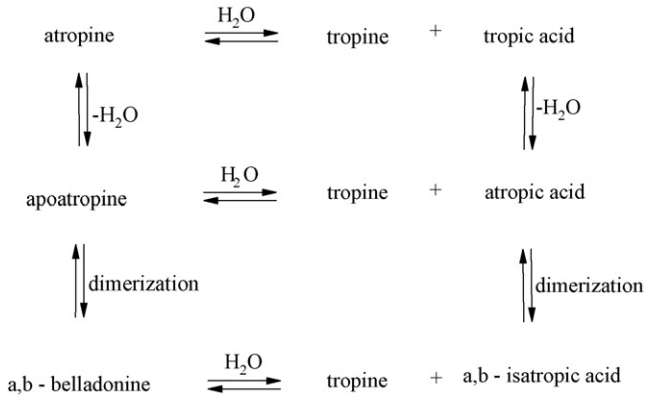
For the last few years, microemulsion electrokinetic chromatography (MEEKC) has become an important field of research in capillary electrophoresis (CE) and provides a wide range of applications [1–10]. MEEKC is an electroseparation technique which uses an aqueous buffer containing minute oil droplets, a surfactant and a co-surfactant to form a microemulsion background electrolyte. The separation mechanism of MEEKC is similar to MEKC (micellar electrokinetic chromatography) which is based on the hydrophobicities and electrophoretic mobilities of the solutes. The different EOF driven migration times of the neutral compounds are primarily governed by the distribution of the uncharged analytes between the aqueous buffer phase and the pseudostationary phase constituted of the oil droplets which are stabilized by both a surfactant and a co-surfactant. For ionic compounds the charged analytes have their own electrophoretic mobility. Thus, the separation observed is a result of both partitioning between the oil droplets and the aqueous phase, and their different electrophoretic mobility [11]. In addition to these effects, electrostatic interaction effects between the analytes and microemulsions play a pivotal role, depending on the relative charges of analytes and surfactant itself [12,13].

Atropine ((±)-hyoscyamine) is a tropane alkaloids being used in ophthalmic diagnosis as mydriatic as well as parasympatholytic, anticholinergic and antiemetic drug. L-Hyoscyamine is formed in several solanaceae [14]. Upon extraction process of the plants material racemization occurs giving atropine [15]. Due to the non-selective isolation process atropine can be accompanied by structurally related substances, i.e. noratropine, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine (scopolamine) and littorine [16], in addition to degradation products. Kirchhof et al. [17] have summarized the total reaction pathway for degradation of atropine in acidic and neu-

Abbreviations: MEEKC, microemulsion electrokinetic chromatography; O/W, oil-in-water; BGE, background electrolyte; MEKC, micellar electrokinetic chromatography; CZE, capillary zone electrophoresis; SDS, sodium dodecyl sulfate; IPC, ion-pair chromatography

* Corresponding author. Tel.: +459 931 8885460; fax: +459 931 8885494.
E-mail address: u.holzgrabe@pharmazie.uni-wuerzburg.de (U. Holzgrabe).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.07.039



Scheme 1. Degradation pathways of atropine in aqueous solution.

tral aqueous solutions (see Scheme 1). Under extreme conditions dimerizations yielding belladonnine and isatropic acid can take place. However, the products are unlikely to occur or to be formed in pharmaceutical preparations of atropine [18]. Due to the lack of a chromophore the degradation product tropine cannot be detected by UV-spectrophotometry, but will appear in the same amount as tropic acid because it is the "other" part of the atropine molecule. However, tropic acid on its part may degrade to give atropic acid. Thus, the amount of tropine can be estimated approximately as the sum of tropic and atropic acid. In any case, apoatropine, tropic acid and atropic acid have to be regarded as the main degradation products of atropine (see Fig. 1). Interestingly enough, the *European Pharmacopoeia* does not consider tropine as an impurity.

Since the stability of atropine is limited, a huge number of ion-pair chromatography (IPC) methods have been reported [e.g. 19,20] including the *European Pharmacopoeia* [16] for determination of atropine degradation products. Kirchhof et al. [17] have employed a simple reversed phase chromatography (RP-HPLC) method using a hydrophilic embedded RP-18 column. Many methods apply a mobile phase gradient and sometimes a flow rate gradient. However, IPC methods especially in combination with gradient elution tend to require a long equilibration time and are often not very robust. MEEKC methods, like other CE techniques (CZE or MEKC), is characterized by a high selectivity. Thus, the aim of this study was to develop an O/W-MEEKC method for the simultaneous separation and determination of atropine, its major degradation products and related substances from the plant materials. The effects of the surfactant SDS and the type and concentration of the additional organic modifier, oil and buffer type, and applied voltage were investigated. A water-in-oil W/O-MEEKC has been tested too and optimized in this study, but only the O/W-MEEKC method

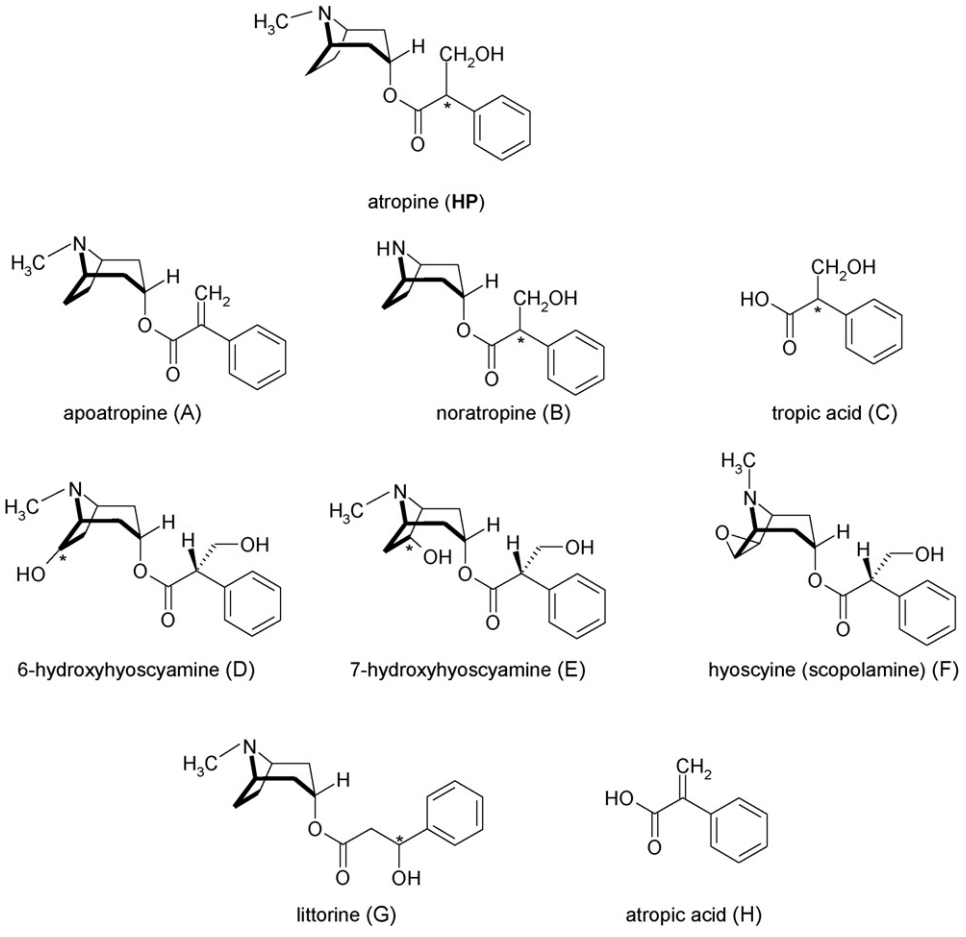


Fig. 1. Structural formulae of atropine and related substances.

was able to successfully separate atropine and all impurities under the optimized conditions. Analysis time was reduced by using a voltage gradient in the last part of the analysis. The established method was validated for the detection and quantification of related substances of atropine sulfate at the 0.1% impurity level.

2. Experimental

2.1. Instrumentations

CE measurements were performed on a HP^{3D}-CE (Agilent Technologies, Waldbronn, Germany) equipped with a DAD detector and the Chemstation 08.03 Software. The detection wavelength was set at 195 nm. Fused-silica capillaries purchased from Polymicro (BGB Analytik, Schloßböckelheim, Germany) with a total length of 48.5 cm, a length to detector is 40.0 cm, and an internal diameter of 50 μm and external diameter of 375 μm were employed. The capillary cartridge was thermo stated at 30 °C.

RP-HPLC was recorded on an Agilent System 1100 LC/MSD (Böblingen, Germany) consisting of a vacuum degasser, a binary pumping system, an autosampler, a thermo stated column compartment, an DAD detector and the LC 3D ChemStation 3D Software (Version 08.04). Thermo Hypersil Aquasil C_{18} analytical column (5 μm particle size, 125 mm × 4 mm i.d.) characterized by a hydrophilic endcapping was purchased from Thermo Hypersil-Keystone, Bellefonte, Pennsylvania, USA.

2.2. Chemicals and materials

Atropine sulfate monohydrate, tropic acid, noratropine, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine (scopolamine) and littorine were provided by Boehringer Ingelheim (Ingelheim, Germany). Apoatropine was synthesized starting from atropine sulfate according to Hesse [21], and atropic acid from tropic acid according to Raper [22]. *n*-Hexane, *n*-heptane, *n*-octane, 1-butanol and 1-pentanol were purchased from Sigma–Aldrich Chemie (Deisenhofen, Germany); ethyl acetate, methanol and isopropanol from Fisher Scientific (Loughborough, UK), acetonitrile from Carl Roth (Karlsruhe Germany), sodium dodecyl sulfate (SDS), sodium octanoate (caprylate) and sodium desoxycholic acid from Fluka (Buchs, Switzerland); sodium tetraborate, tris-(hydroxymethyl)-amino methane, sodium dihydrogenphosphate, sodium hydroxide, boric acid and orthophosphoric acid 85% from Grüssing (Fillsum, Germany). All samples and buffers were prepared using ultrapure-Milli-Q water (Millipore, Milford, MA, USA), than filtered through a 0.22 μm filter (Carl Roth GmbH, Karlsruhe, Germany) prior to use.

2.3. Methods and conditions

2.3.1. O/W-MEEKC methods

The samples were injected at the anodic end of the capillary by pressure of 50 mbar for 5 s. The separations were performed using 10 mM sodium borate buffer (pH 9.2 ± 0.1) or 20 mM phosphate buffer (pH 2.0 ± 0.1) in the running buffer. The pH of the sodium tetraborate buffer and phosphate buffer were adjusted with 20 mM boric acid or 20 mM sodium hydroxide and 50 mM phosphoric acid, respectively. The optimized O/W microemulsion BGE solution was prepared by weighing 0.8 g octane, 6.62 g 1-butanol, 2.0 g 2-propanol, 4.44 g SDS and 86.14 g 10 mM sodium tetraborate buffer pH 9.2 in a flask. The solution was sonicated for 20 min to aid dissolution and to form an optically transparent microemulsion. The other microemulsions used during the optimization process were prepared in a similar manner. If another organic solvent (modifier) was used, they were added after the co-surfactant 1-butanol. The positive separation voltages (12–20 kV) were applied when using the tetraborate buffer pH 9.2 and negative voltage (−15 kV) in the case of the phosphate buffer pH 2.0.

New capillaries were conditioned at 40 °C rinsing with 0.1 M NaOH for 10 min, with water for 5 min, with 0.1 M H_3PO_4 for 10 min and water for 5 min. Before running a series of experiments, the capillary was conditioned at 30 °C rinsing with 0.1 M NaOH for 5 min, with water for 5 min and the microemulsion background electrolyte (BGE) for 10 min. Between each run, the capillary was rinsed at 30 °C with 0.1 M NaOH for 3 min, with MeOH for 3 min, with 0.1 M H_3PO_4 for 2 min and water for 2 min and conditioned with BGE solution for 6 min. At the end of a working day, the capillary was rinsed at 30 °C with 0.1 M NaOH for 10 min, water for 5 min and methanol for 15 min. Capillary wash cycles were performed at a pressure of approximately 2.0 bar.

2.3.2. W/O-MEEKC method

The optimized W/O microemulsion were prepared by adding 2% (w/w) octane, 78% (w/w) 1-butanol, 10% (w/w) SDS and 10% (w/w) 70 mM tris-phosphate buffer, pH 8.0. The order of the addition of the microemulsion compositions was found to be important in the formation of the microemulsion. Initially the surfactant (SDS) was mixed with the oils (octane and 1-butanol), and then the aqueous buffer was added. This mixture was sonicated for 20 min to aid dissolution and to form an optically transparent microemulsion. Subsequently 10% (w/w) organic modifier (MeOH or ACN) was added. This mixture was again sonicated for 10 min. The samples were hydrodynamically injected at 50 mbar for 5 s, and separations performed at 25 °C using a constant voltage of −30 kV in the reserved polarity mode.

2.3.3. RP-HPLC method

The HPLC method, its chromatographic conditions and gradient elution for the separation of atropine and related substances was performed as described by Kirchhof et al. [17].

2.4. Sample preparation

Test solutions during the optimization of the method were prepared by dissolving the appropriate weights of atropine sulfate and each impurity in 10.0 ml in the final microemulsion BGE. This solution was sonicated for 10 min, and than diluted with the same BGE solution.

2.4.1. Preparation of standard solutions

Stock solutions of atropine sulfate and each related substance were prepared by dissolving 2.0 g atropine sulfate monohydrate, 2.0 mg tropic acid, 3.0 mg apoatropine, 2.0 mg atropic acid, 4.0 mg noratropine, 2.0 mg 6-hydroxyhyoscyamine, 2.0 mg 7-hydroxyhyoscyamine, 2.0 mg hyoscine and 2.0 mg littorine, respectively, in 100 ml of the microemulsion BGE solution. Then 10.0 ml of each solution was diluted to 100 ml with the same BGE solution. The samples of atropine during the validation of the method spiked with different levels of the impurities were prepared by mixing corresponding amounts of the atropine and each impurity stock solution.

3. Results and discussion

3.1. Method optimization

3.1.1. Standard MEEKC separation

A common microemulsion solution [23] consisting of 0.8% (w/w) octane as the oil droplet phase, 6.62% (w/w) 1-butanol as co-surfactant, 3.33% (w/w) SDS as surfactant to stabilize the microemulsion droplets and 89.25% (w/w) aqueous buffer medium was initially used to separate atropine its major degradation products (**A**, **C** and **H**) and all other impurities. Sodium tetraborate buffer was chosen as a high pH buffer due to the greater magnitude of electroosmotic flow (EOF), providing an acceptable analysis time. Typical electropherograms of the standard separation are shown in Fig. 2.

To achieve a baseline separations (Fig. 2b) between tropic acid (**C**) and scopolamine (**F**), and atropine (**HP**) and noratropine (**B**) on the one hand and between atropine and littorine (**G**) on the other hand, all parameters affecting the separation, such as the applied voltage, type and concentration of additional organic modifier and surfactant, pH buffer and type of the droplet oil phase and co-surfactant have been studied. The resolution factors between tropic acid and the next peak (in this case scopolamine $R_{\mathbf{F,C}}$), noratropine and atropine $R_{\mathbf{HP,B}}$ and atropine and littorine $R_{\mathbf{G,HP}}$ as well as the migration time of the last peak (apoatropine) $\mathrm{MT}_{\mathbf{A}}$ in the electropherogram especially were considered during the optimization procedure.

3.1.2. Effect of operating voltage

Increasing the voltage in a range of 12–20 kV decreased the migration time of the test solution and increased the operating current generated. Currents of 128 and 46 μA were generated at the 20 and 12 kV, respectively. A voltage of 15 kV gives an appropriate resolution between tropic acid and scopolamine according to the short analysis time, and was, thus, selected for the further optimization (data not shown).

3.1.3. Effect of organic modifier

The addition of solvents to the microemulsion BGE solution diminishes the interactions between analytes and the pseudo-stationary phase, and increases their solubility in the aqueous phase [24,25]. Isopropanol can act in a similar way as a co-surfactant and helps to stabilize the system. Therefore, it can be added at higher concentrations without destroying the microemulsion BGE solution compared with methanol or acetonitrile [23,26]. The effects of the organic modifier type (isopropanol, methanol and acetonitrile) and their concentration ranging from 0 to 5% (w/w) on the MEEKC separation have been studied. In all cases, the microemulsion BGE solutions were prepared by adding various volumes of the organic solvent to the microemulsion compositions; thus, the increase in the content of organic solvent means a reduction in the amount of tetraborate buffer. Table 1 summarizes the data affected by adding different percentage of isopropanol, methanol or acetonitrile as organic modifier. As the isopropanol concentration increases the

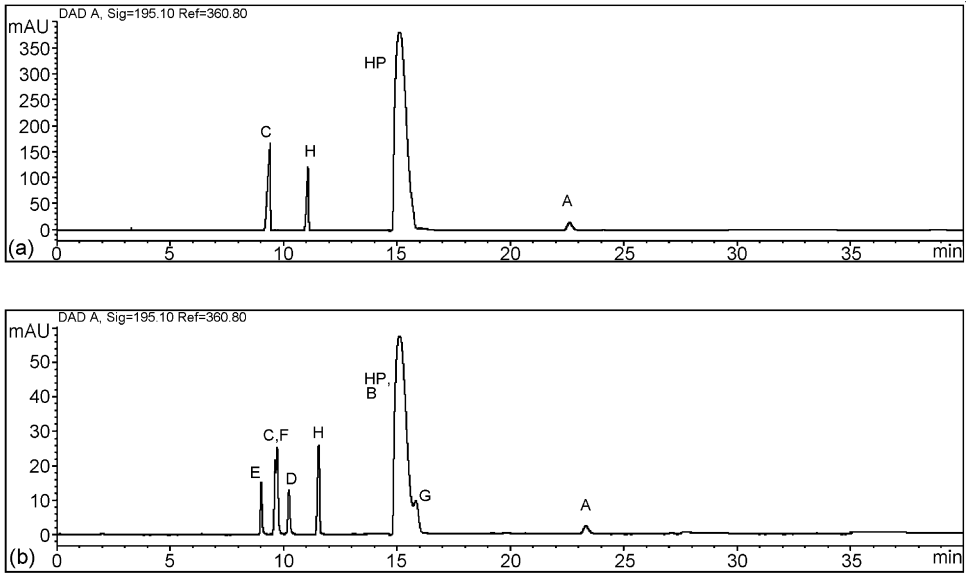


Fig. 2. Standard MEEKC separation of: (a) atropine and its major degradation products; (b) atropine and related substances. Separation conditions: BGE; 0.8% (w/w) octane, 6.62% (w/w) 1-butanol, 3.33% (w/w) SDS, 89.25% (w/w) 10 mM sodium tetraborate buffer pH 9.2, applied voltage +18 kV, temperature 30 °C, detection wavelength 195 nm, capillary fused-silica capillary (50 μm i.d. ×40 cm length). For the assignment of the compounds see Fig. 1.

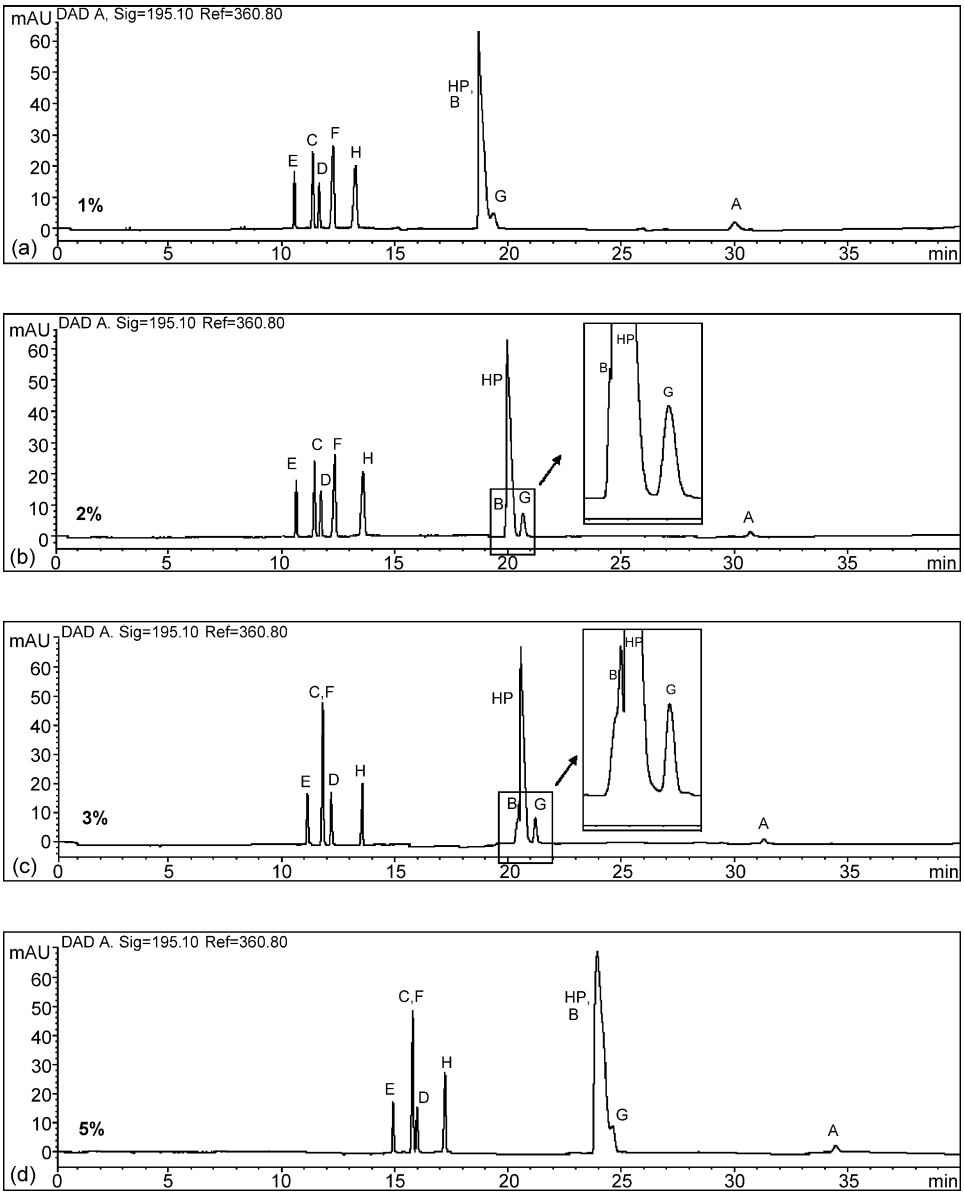


Fig. 3. Effect of isopropanol addition on the MEEKC separation of atropine and related substances. Separation conditions: BGE; 0.8% (w/w) octane; 6.62% (w/w) 1-butanol; *x*% (w/w) isopropanol: (a) 1%, (b) 2%, (c) 3%, (d) 5%; 3.33% (w/w) SDS; (89.25 − *x*)% (w/w) 10 mM sodium tetraborate buffer pH 9.2; applied voltage +15 kV. Other conditions: see Fig. 2. For the assignment of the compounds see Fig. 1.

polarity of the microemulsion BGE solution decreases and the solubility of the hydrophobic compounds in the aqueous phase increases. Consequently, their effective mobilities also change and a reversed migration order between scopolamine (**F**) and 6-hydroxyhyoscamine (**D**) and the baseline separation between atropine (**HP**) and littorine (**G**) were observed (see Fig. 3). In addition, the migration times of the analytes increased with higher percentages of the isopropanol or other organic solvents (methanol, acetonitrile), which can be explained by a slower EOF due to the reduction of zeta potential [27]. The addition of 2% (w/w) isopropanol shows a good separation (Fig. 3b) of the atropine related substances. However, no resolution between atropine (**HP**) and noratropine (**B**) could be achieved.

Using methanol or acetonitrile gives a reverse of migration order between tropic acid (**C**) and 7-hydroxyhyoscamine (**E**) in addition to a longer migration time. However, 2% (w/w) isopropanol was chosen for further optimization because of the better overall separation observed at this level.

3.1.4. Effect of surfactant

First, the effect of SDS concentration on the separation was examined in a range of 3.3–5.0% (w/w). Increasing the SDS concentration increases the ionic strength of buffers, which reduces the EOF level and increases the analysis time. Higher concentrations of SDS increase the retention factor of neutral compounds (at pH 9.2) due to increased charge density on the oil droplets. A reversed migration order between 6-hydroxyhyoscamine (**D**) and scopolamine (**F**) and the baseline separation between atropine (**HP**) and noratropine (**B**) were observed at the concentration level 4.44% (w/w). Thus, 4.44%

Table 1
The data of additional organic modifier effects on the separation response (see Section 3.1.3)

	Percentage (w/w)				
	0	1	2	3	5
Isopropanol					
$R_{D,C}$	–	1.32	1.31	–	–
$R_{HP,B}$	–	–	0.2	0.4	–
$R_{G,HP}$	0.61	0.35	1.37	1.65	0.31
MT_A	28.86	30.05	30.81	31.46	34.54
Methanol					
$R_{E,C}$	–	1.17	1.26	1.35	1.14
$R_{HP,B}$	–	–	–	–	–
$R_{G,HP}$	0.61	1.18	1.18	1.22	1.20
MT_A	28.86	29.21	30.25	32.45	37.76
Acetonitrile					
$R_{E,C}$	–	0.89	1.05	2.32	2.08
$R_{HP,B}$	–	–	–	0.95	1.01
$R_{G,HP}$	0.61	1.28	1.39	1.96	1.95
MT_A	28.86	45.58	46.42	48.65	56.48

(w/w) SDS was selected for further optimization (data not shown).

Second, the type of the surfactant of the MEEKC separation have also been studied, as it influences the level and direction of the EOF, the charge and size of the oil droplets and ion pairing in the system. Two anionic surfactants sodium caprylate and sodium deoxycholate have been used in a concentration of 3.33% (w/w) to replace SDS. The separation of atropine and only its degradation products (apoatropine, tropic- and atropic acid) with sodium deoxycholate gave better resolution and shorter analysis time compared with sodium caprylate or SDS (cf. Figs. 2 and 4). However for atropine and all impurities, the migration orders vary more significantly in both cases sodium caprylate and sodium deoxycholate, and in addition gave a poor peak shape. Therefore, SDS was determined to be the optimal surfactant.

3.1.5. Effect of low-pH buffer

The effect of acidic conditions (pH 2.0) on the MEEKC separation of atropine and degradation products and related substances has been studied applying a reversed polarity and using a phosphate puffer (20 mM, pH 2.0) as the aqueous buffer medium in the microemulsion BGE solution. Under this acidic condition the EOF is suppressed and the neutral analytes depend on a chromatographic-type separation mechanism; the resolution of the analytes is exclusively driven by their selective distribution between the negatively charged oil droplets moving to the anode (detector), and the aqueous phase. As a consequence, under these reversed-flow conditions the neutral compounds (at pH 2) strongly partitioned into the oil droplets (lipophilic compounds) and, thus, migrate faster than the less partitioned compounds (hydrophilic analytes) [28]. Under these conditions a full baseline separation of all impurities could not be achieve, although a high SDS concentration of 6.0% (w/w) was employed.

3.1.6. Effect of oil and co-surfactant type

In general, *n*-octane and *n*-heptane are used to form the oil droplet phase in microemulsion BGE solution [23,24,29]. The effect of the choice of oil type on the separation of atropine related substances were examined. Although, similar migration times were obtained when *n*-hexane, *n*-heptane and *n*-octane were tested, 6-hydroxyhyoscyamine (**D**) migrated faster than scopolamine (**F**) and the resolution between atropine (**HP**) and noratropine (**B**) decreased as the carbon numbers of the oil used decreased. Using ethyl acetate the migration times of the analytes were slightly shorter than the ones using other oils because of the lower carbon content in ethyl acetate (data not shown).

The effect of co-surfactant type of the separation has been studied too. A butanol microemulsion BGE showed better

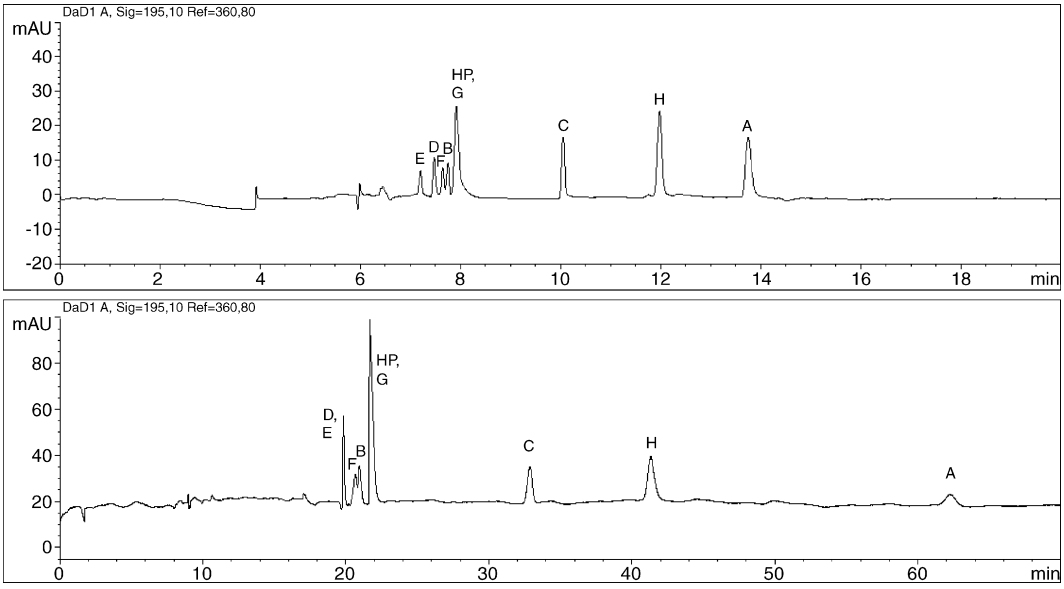


Fig. 4. Effect of surfactant type on the MEEKC separation of atropine and related substances. Surfactant types: (a) sodium deoxycholate; (b) sodium caprylate. Other conditions: see Fig. 2. For the assignment of the compounds see Fig. 1.

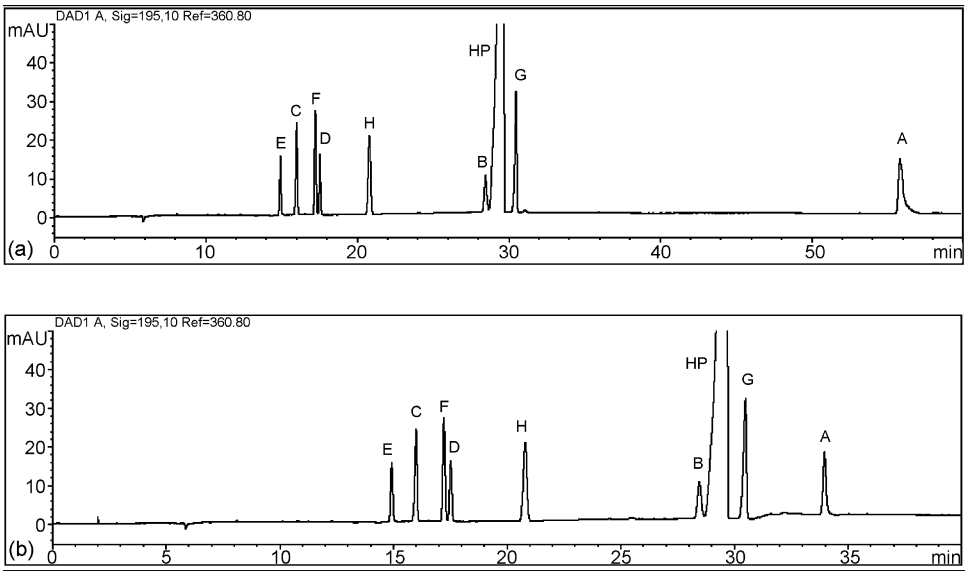


Fig. 5. Electropherograms of atropine and all impurities under optimized MEEKC conditions: BGE; 0.8% (w/w) octane, 6.62% (w/w) 1-butanol, 2% (w/w) 2-propanol, 4.44% (w/w) SDS, 86.14% (w/w) 10 mM sodium tetraborate buffer pH 9.2; applied voltage: (a) isocratic +15 kV and (b) gradient voltage starting isocratically an +15 kV for 31 min, increasing the applied voltage to +25 kV till 32 min, and carried out again isocratically to 35 min. Other conditions: see Fig. 2. For the assignment of the compounds see Fig. 1.

selectivity of the separation than a pentanol microemulsion BGE. Butanol has a lower viscosity and therefore generates a significant current compared to pentanol.

3.1.7. Analysis time optimization

Successful MEEKC separation of atropine and all impurities was achieved using a microemulsion BGE solution consisting of 0.8% (w/w) octane, 6.62% (w/w) 1-butanol, 2.0% (w/w) 2-propanol, 4.44% (w/w) SDS and 86.14% (w/w) 10 mM sodium tetraborate pH 9.2 and an applied voltage of +15 kV. Using these conditions a long analysis time was observed due to the slow migration of apoatropine (**A**). In order to speed up the apoatropine migration a voltage gradient was applied after the migration of littorine characterized by an increase of the applied voltage for 0.5 min from +15 to +25 kV (see Fig. 5). Hence, the analysis time reduces to 35 min versus 60 min.

3.1.8. W/O-MEEKC separation

Water-in-oil microemulsions have interesting potential for the separation of water insoluble compounds. Altria et al. [30] investigated a water-in-oil W/O-MEEKC method for neutral and acidic compounds separated. A similar W/O microemulsion has been applied in this study for the separation of atropine and related substances. The W/O microemulsion BGE solution consisted of 2% (w/w) octane, 78% (w/w) 1-butanol, 10% (w/w) SDS and 10% (w/w) 70 mM tris-phosphate buffer pH 8.0. W/O-MEEKC generates a low separation current. Thus, high buffer concentrations are needed in W/O-MEEKC to generate sufficient operating current for stable and efficient resolutions to be achieved. The organic modifier partitions in both the aqueous phase and the oil phase of the microemulsion. Methanol or acetonitrile were added at a percentage of 10% (w/w) to the final microemulsion BGE solution described above to increased the resolution between the lipophilic compounds, e.g. atropine, noratropine, 6-hydroxyhyoscyamine and 7-hydroxyhyoscyamine. Fig. 6 shows the optimized separation of atropine related substances with W/O-MEEKC. Addition of methanol gave a better separation according to the peak form, but a noisy baseline compared to the addition of acetonitrile. However, in both cases the baseline separation between 6-hydroxyhyoscyamine (**D**) and 7-hydroxyhyoscyamine (**E**) could not be achieved.

3.2. Method validation

The final O/W-MEEKC method, which was employed for the determination of atropine and related substances (Fig. 7), was validated according to the International Conference on Harmonization (ICH) guidelines Q2A [31] and Q2B [32] as well as the *European Pharmacopoeia* [16] with respect to specificity, linearity, range, limit of quantification and detection, precision, accuracy and robustness. Tropic acid was used as an external standard, at a concentration of 5.0 μg/ml in order to compensate fluctuations of the migration times.

3.2.1. Specificity, linearity, LOD, and LOQ

With respect to specificity, all relevant impurities of atropine were well baseline separated (see Fig. 7). By spiking the atropine sample with the individual impurities, the peaks of the electropherogams were assigned. In order to determine the impurity level in samples of the atropine sulfate, a calibration was performed using the corrected peak area ratio method for eight concentration levels of each impurity in the range of 0.15–20 μg/ml for tropic acid and atropic acid, 0.20–25 μg/ml for 6-hydroxyhyoscyamine and 7-hydroxyhyoscyamine, 0.30–35 μg/ml for scopolamine and littorine, and 0.35–40 μg/ml for noratropine and apoatropine, corresponding to a 0.015–2.0%, 0.02–2.5%, 0.03–3.5% and

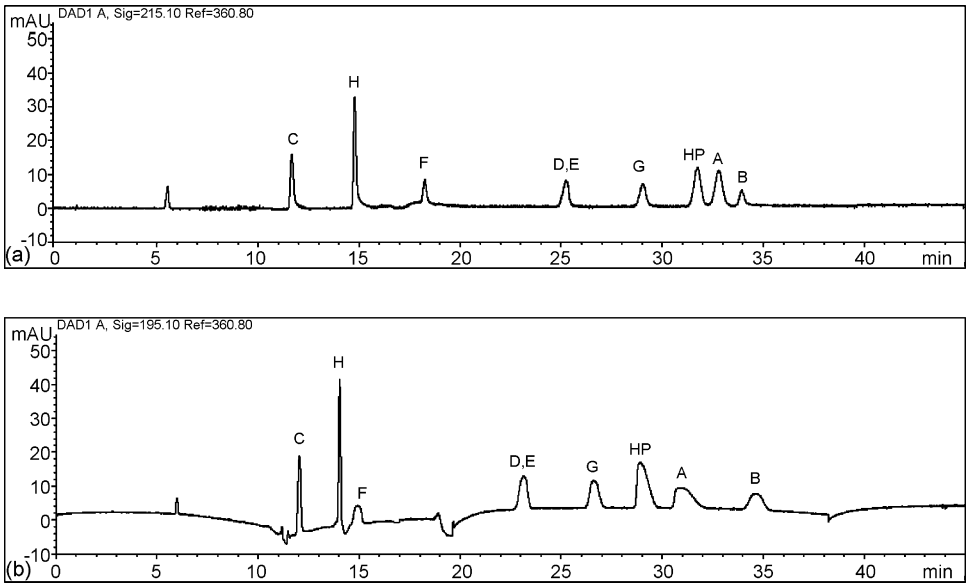


Fig. 6. W/O-MEEKC separation of atropine related substances. Separation conditions: microemulsion; 2% (w/w) octane, 78% (w/w) 1-butanol, 10% (w/w) SDS, 10% (w/w) 70 mM tris-phosphate buffer pH 8.0, BGE 90% (w/w) microemulsion and (a) 10% (w/w) methanol; (b) 10% (w/w) acetonitrile, applied voltage −30 kV (reversed polarity), temperature 25 °C. Other conditions: see Fig. 2. For the assignment of the compounds see Fig. 1.

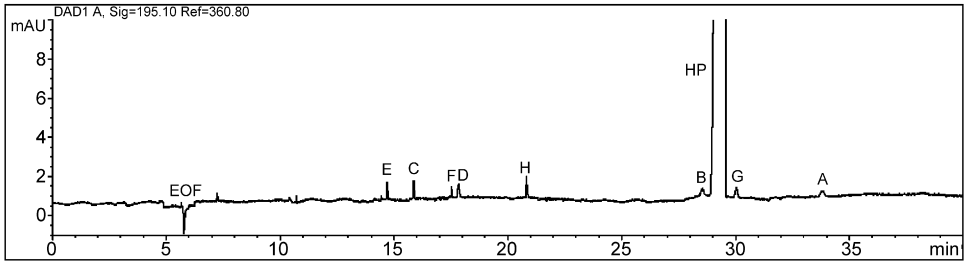


Fig. 7. Electropherograms of atropine sulfate spiked with 0.1% of the impurities. For experimental conditions see Fig. 5. For the assignment of the compounds see Fig. 1.

0.035–4.0% content of the impurity, respectively, in an atropine sulfate sample of 1.0 mg/ml. Each standard was injected three times randomized and the mean corrected peak area of each impurity was evaluated. Calibration curves were obtained by plotting the concentration levels of the impurities against the peak-area ratios. The data are summarized in Table 2. In all cases, straight regression lines with correlation coefficients (r) above 0.997 were obtained. The intercept values were not significantly different from zero (96% confidence). The LOD and LOQ were investigated according to *European Pharmacopoeia* [16], i.e. a signal-to-noise ratio of 3:1 and 10:1, respectively (see Table 2).

3.2.2. Precision and accuracy

The precision of the method was investigated with respect to repeatability and intermediate precision. Two different concentrations of the impurities of 1.0 and 2.0 μg/ml corresponding to the 0.1% and 0.2% impurity level, respectively, based on a con-

Table 2
Calibration data, LOD and LOQ of the impurities

Impurity	Range (μg/ml)	Slope	Intercept	r	LOD		LOQ	
					μg/ml	%	μg/ml	%
E	0.20–25	0.2465	0.013	0.9992	0.10	0.01	0.20	0.02
C	0.15–20	0.253	0.0143	0.9986	0.08	0.01	0.15	0.02
F	0.30–35	0.181	0.0013	0.9981	0.15	0.02	0.30	0.03
D	0.20–25	0.2185	0.0186	0.9995	0.10	0.01	0.20	0.02
H	0.15–20	0.2322	0.0471	0.9982	0.08	0.01	0.15	0.02
B	0.35–40	0.1316	0.0407	0.9977	0.20	0.02	0.35	0.04
G	0.30–35	0.1369	0.0419	0.9974	0.15	0.02	0.30	0.03
A	0.35–40	0.0886	−0.0127	0.9979	0.20	0.02	0.35	0.04

Table 3
Intra-day and inter-day precision of the corrected peak area ratios and relative migration times at two impurity levels of atropine sulfate

	Intra-day precision ($n = 6$)				Inter-day precision ($n = 18$)			
	Area ratio	R.S.D. (%)	RMT (min)	R.S.D. (%)	Area ratio	R.S.D. (%)	RMT (min)	R.S.D. (%)
Impurity (0.1%)								
E	0.183	1.29	0.94	0.12	0.189	1.78	0.92	1.05
C	0.242	2.45	1.01	0.78	0.254	2.45	1.00	1.54
F	0.155	1.19	1.08	0.91	0.151	0.98	1.10	2.64
D	0.200	1.98	1.11	1.09	0.204	2.68	1.15	1.98
H	0.177	2.08	1.31	0.15	0.185	3.15	1.33	3.46
B	0.110	2.73	1.79	1.06	0.113	2.79	1.69	2.67
G	0.130	1.66	1.92	0.77	0.139	1.65	1.91	3.89
A	0.062	2.23	2.14	1.19	0.069	3.41	2.16	3.42
Impurity (0.2%)								
E	0.361	1.17	0.96	0.54	0.372	2.14	0.91	2.17
C	0.364	1.92	1.01	0.60	0.361	2.69	1.05	1.96
F	0.256	0.58	1.09	0.36	0.259	3.04	1.14	1.54
D	0.356	0.25	1.11	1.56	0.366	1.65	1.18	0.96
H	0.396	0.96	1.31	1.15	0.401	1.25	1.36	2.69
B	0.220	1.44	1.80	0.89	0.218	2.56	1.75	3.45
G	0.236	1.29	1.92	1.22	0.231	2.78	1.98	2.09
A	0.129	2.74	2.15	1.15	0.136	2.91	2.19	4.02

centration of atropine sulfate of 1.0 mg/ml were prepared. Each solution was injected six times on one day (intra-day precision) and on three different days (inter-day precision). The relative migration times (RMT) of each impurity to the external standard (tropic acid) as well as the corrected peak area ratio are summarized in Table 3. The acceptable relative standard deviations (R.S.D.) were found with respect to the relative migration times for intra-day and inter-day precision at the impurity levels of 0.1%, and 0.2%. The same holds true for the corrected peak area-ratio R.S.D. values for the intra-day and inter-day precision at the impurity levels of 0.1% and 0.2%.

The accuracy of the method was investigated in a similar manner by injecting samples at two different concentrations of each impurity at about 1.0 and 2.0 μg/ml which corresponds to 0.1% and 0.2%, respectively, in an atropine sulfate solution of 1.0 mg/ml. The data summarized in Table 4, show acceptable accuracy of the method. All accuracy values (R.S.D. between be 2.4% and 3.7%) are significant lower than the ±5% of the nominal values.

3.2.3. Robustness

Robustness relates to the capacity of a method to remain unaffected by small variations of the operation parameters such as variation of the applied voltage ±1 kV, the capillary temperature ±1 °C, the pH of the puffer ±0.1, the buffer concentration ±1 mM and the microemulsion composition ±0.1% (w/w), con-

Table 4
Accuracy of the method tested at 0.1% and 0.2% impurity levels of atropine sulfate

	Intra-day accuracy ($n = 6$)			Inter-day accuracy ($n = 18$)		
	Nominal concentration (μg/ml)	Accuracy (%)	R.S.D. (%)	Nominal concentration (μg/ml)	Accuracy (%)	R.S.D. (%)
Impurity (0.1%)						
E	1.01	102.4	1.08	1.04	101.5	2.03
C	1.00	99.6	2.36	0.99	99.1	1.65
F	1.17	98.2	2.22	1.11	98.9	0.99
D	1.08	101.6	1.15	1.12	102.1	2.07
H	1.01	103.7	0.98	0.97	102.9	1.54
B	1.10	100.1	1.19	1.12	100.3	3.69
G	0.98	98.7	1.02	1.00	99.2	2.66
A	1.04	97.9	1.94	1.02	97.2	2.81
Impurity (0.2%)						
E	2.02	101.3	1.69	2.00	102.1	2.92
C	2.00	100.6	0.78	2.01	104.6	2.54
F	1.90	104.8	2.15	1.96	104.9	1.69
D	2.16	99.6	1.10	2.15	100.1	3.12
H	2.10	102.6	2.09	2.06	101.6	1.56
B	1.95	98.1	1.32	1.99	98.5	2.91
G	2.12	98.5	1.12	2.09	98.3	2.14
A	2.07	99.4	2.07	2.09	98.1	1.17

Table 5
Comparison of the MEEKC method with HPLC method of the impurity test of atropine sulfate at the level 0.2%

Impurity 0.2 (%)	Concentration (μg/ml)	MEEKC content (%)	R.S.D. (%) ($n=6$)	HPLC content (%)	R.S.D. (%) ($n=6$)
E	2.02	101.3	1.69	99.8	0.97
C	2.00	100.6	0.78	100.7	0.35
F	1.90	104.8	2.15	104.6	1.12
D	2.16	99.6	1.10	100.0	0.65
H	2.10	102.6	2.09	101.9	0.78
B	1.95	98.1	1.32	98.4	1.05
G	2.12	98.5	1.12	98.9	1.01
A	2.07	99.4	2.07	98.7	1.20

sidering the relative migration time (RMT) of the last peak and the resolution factors (*R*s) between tropic acid and scopolamine $R_{\mathbf{F,C}}$, noratropine and atropine $R_{\mathbf{HP,B}}$ and atropine and littorine $R_{\mathbf{G,HP}}$.

The solution, containing of 1.0 mg/ml of atropine sulfate spiked with 0.1% of the impurities, was analysed six times under either condition. The data of robustness with respect to relative migration time and resolutions are summarized and displayed in Fig. 8. The relative migration time of the last peak varied between 2.03 and 2.22 corresponding to relative variations of +4.69% and −4.23%, respectively, in comparison to the standard conditions. The resolution factors of the critical peak pair $R_{\mathbf{F,C}}$, $R_{\mathbf{HP,B}}$ and $R_{\mathbf{G,HP}}$ varied more significantly in a range of 1.52 and 2.43 (see Fig. 8).

Hence, acceptable relative variations of the relative migration time (RMT) and resolution values (*R*s) with remaining baseline separation were found. Thus, the method can be considered to be very robust against small variations of the standard conditions.

3.3. Comparison of the MEEKC-Method with HPLC

The established O/W-MEEKC method was applied to determine the related substances of atropine sulfate. For the purpose of comparison, the same sample was analyzed using the "traditional" RP-HPLC method reported by Kirchhof et al. [17]. The results of the quantitation of all impurities of atropine sulfate in the test solution 0.2% are given in Table 5. However, a good agreement was obtained between the MEEKC method and HPLC method. Although, in case of precision, the relative standard deviations (R.S.D.) of the MEEKC method was not as good as the R.S.D. seen with HPLC method, the selectivity of established MEEKC method was better than with HPLC method.

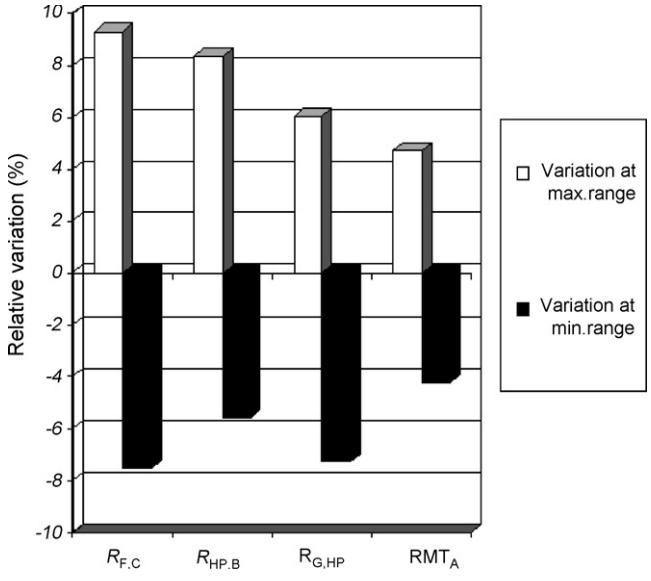


Fig. 8. Relative variation of the resolution factors $R_{\mathbf{F,C}}$, $R_{\mathbf{HP,B}}$ and $R_{\mathbf{G,HP}}$ as well as relative migration time of the last peak $RMT_{\mathbf{A}}$ at small variations of the standard conditions.

4. Conclusions

An oil-in-water MEEKC method characterized by an excellent baseline separation of all compounds of atropine has been developed and validated for the evaluation of related substances of atropine sulfate. Applying a reversed polarity the separations at a low-pH background electrolyte microemulsion or an water-in-oil MEEKC method have been studied but gave separations of lower quality than the O/W MEEKC method.

The method in *European Pharmacopoeia* for determination of all impurity using ion-pair RP-HPLC requires a high amount of ion-pair reagent in the mobile phase and applied a gradient system. IPC method tends to be not very robust and show limited selectivity. The MEEKC method is specific and robust allowing the detection and quantification of all impurities at concentrations of at least 0.02% relative to atropine sulfate at a concentration of the test solution of 1.0 mg/ml. In addition, the method is relatively inexpensive due to low consumption of chemicals and sample compounds. Overall, CE and its related separation techniques CZE, CCE, MEKC and MEEKC should also be considered more often when developing pharmaceutical monographs in the International Pharmacopoeias.

Acknowledgements

Thanks are due to the Boehringer Ingelheim, Germany, for providing atropine sulfate samples and related natural compounds, Dr. E. Heller for synthesizing atropic acid and apoatropine, and the Syrian government for providing a grant to Y. Bitar.

References

[1] Y. Mrestani, N. El-Mokdad, H.H. Ruettinger, R.H.H. Neubert, Electrophoresis 19 (1998) 2895–2899.

[2] M.F. Miola, M.J. Snowden, K.D. Altria, J. Pharm. Biomed. Anal. 18 (1998) 785–797.
[3] J.P. Quirino, S. Terabe, K. Otsuka, J.B. Vincent, G. Vigh, J. Chromatogr. A 838 (1999) 3–10.
[4] J.M. Sánchez, V. Slavadó, J. Chromatogr. A 950 (2002) 241–247.
[5] S.E. Lucangioli, C.N. Carducci, S.L. Scioscia, A. Carlucci, C. Bregni, E. Kenndler, Electrophoresis 24 (2003) 984–991.
[6] A. Marsh, K.D. Altria, B.J. Clark, Electrophoresis 259 (2004) 1270–1278.
[7] M.D. Mertzman, J.P. Foley, Electrophoresis 25 (2004) 723–732.
[8] Q. Li, C.K. Chang, C.W. Huie, Electrophoresis 26 (2005) 3349–3359.
[9] S.H. Hansen, Z.A. Sheribah, J. Pharm. Biomed. Anal. 39 (2005) 322–327.
[10] S. Furlanetto, S. Orlandini, A.M. Marras, P. Mura, S. Pinzauti, Electrophoresis 27 (2006) 805–818.
[11] J. Strasters, M. Khaledi, Anal. Chem. 63 (1991) 2503–2508.
[12] S.H. Hansen, C. Gabel-Jensen, D.T.M. El-Sherbiny, S. Perdersen-Bjergaard, Trends Anal. Chem. 20 (2001) 614–619.
[13] C.W. Klampf, Electrophoresis 24 (2003) 1537–1543.
[14] D. Frohne, H.J. Pfänder, Giftpflanzen, 4th ed., Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1997.
[15] A.A. Al-Badr, F.G. Muthadi, in: K. Florey (Ed.), Analytical Profiles of Drug Substances, vol. 14, Academic Press, Orlando, 1985, pp. 340–389.
[16] European Pharmacopoeia, 5th ed., Council of Europe, Strasbourg, France, 2005.
[17] C. Kirchhof, Y. Bitar, S. Ebel, U. Holzgrabe, J. Chromatogr. A 1046 (2004) 115–120.
[18] W. Lund, T. Waaler, Pharm. Acta Helv. 45 (1970) 701–707.
[19] N.D. Brown, H.K. Sleeman, J. Chromatogr. 150 (1978) 225–228.
[20] B. Kreilgard, Arch. Pharm. Chem., Sci. Ed. 6 (1978) 109–115.
[21] O. Hesse, Liebigs Ann. Chem. 261 (1891) 87–107.
[22] H.S. Raper, J. Chem. Soc. (1923) 2557–2559.
[23] K.D. Altria, B.J. Clark, P.-E. Mahuzier, Chomatographia 52 (2000) 758–768.
[24] M. Rodríguez, M. Sánchez, V. González, F. García, Anal. Chem. Acta 298 (1994) 423–430.
[25] S. Frías, M. Sánchez, M. Rodríguez, Anal. Chem. Acta 503 (2004) 271–278.
[26] S. Pedersen-Bjergaard, O. Naess, S. Moestue, K. Rasmussen, J. Chromatogr. A 876 (2000) 201–211.
[27] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801–1807.
[28] R. Pomponio, R. Gotti, B. Luppi, V. Cavrini, Electrophoresis 24 (2003) 1658–1667.
[29] K. Altria, J. Chromatogr. A 892 (2000) 171–186.
[30] K.D. Altria, M.F. Broderick, S. Donegan, J. Power, Electrophoresis 25 (2004) 645–652.
[31] Guidelines Q2A, Validation of Analytical Procedures, Methology, International Conference on Harmonization 1996, available at: http://www.ich.org.
[32] Guidelines Q2B, Validation of Analytical Procedures, Methology, International Conference on Harmonization 1996, available at: http://www.ich.org.