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Separation of antipsychotic drugs (clozapine, loxapine) and their metabolites by capillary zone electrophoresis

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Abstract

Two antipsychotic drugs (clozapine and loxapine) and six metabolites, *N*-demethylclozapine, clozapine *N*-oxide, *N*-demethylloxapine (amoxapine), 7-hydroxyloxapine, 8-hydroxyloxapine, 8-hydroxyamoxapine, were separated by capillary zone electrophoresis. Variation of pH and ionic strength of the acidic phosphate buffer (pH below 4) did not enable the separation of loxapine and one of its metabolites. Resolution of the single parent drugs and their metabolites was possible in background electrolytes (phosphate, pH 3.5, 60 mmol/l) containing either 0.2% (w/v) polyvinylpyrrolidone as replaceable pseudo-stationary phase, or 0.75 mmol/l β-cyclodextrin added as complex-forming agent. Full separation of the mixture with baseline resolution of all analytes was obtained with a background electrolyte with heptakis-6-sulfato-β-cyclodextrin added as negatively charged complexation agent with improved separation selectivity.

Keywords: Pharmaceutical analysis; Clozapine; Loxapine

1. Introduction

Until the end of the 1960s, classical antipsychotics like haloperidol and chlorpromazine, were used in therapy to treat the positive symptoms of schizophrenia [1], a debilitating disorder of the central nervous system. In the early 1970s, clozapine [2] was administered to patients who did not respond to classical antipsychotics. Clozapine, an atypical antipsychotic, is used for the treatment of the positive and the negative symptoms of schizophrenia as well [3]. It was found, on the other hand, that it induces agranulocytosis [4,5], which can be fatal in some cases. This fact led to its withdrawal from the USA market and the limitation of its use in Europe. During 1990, clozapine was made available in the USA through a monitoring system designed to minimize the risk of agranulocytosis [6]. Some authors suggested that the main cause of this side effect was *N*-demethylclozapine rather than the parent drug [7], which is one of its main metabolites together with clozapine *N*-oxide. It is evident that an accurate monitoring of the drug and the biotransformed products is needed during the period of treatment, in order to reduce the probability of causing such severe side effects.

Agranulocytosis is scarcely or not occurring using loxapine, a dibenz[1,4]oxazepine, which displays some structural and pharmacological similarities with clozapine, but may cause other side effects [8]. Like clozapine, it is rapidly absorbed following oral

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administration. It is extensively metabolized in the liver, undergoing several phase I reactions, including *N*-demethylation (forming amoxapine), *N*-oxidation on the 4-piperidinyl nitrogen atom (forming the *N*-oxide) and aromatic hydroxylation (forming 7-hydroxy- and 8-hydroxyloxapine) [8]. Amoxapine, and some of the phenolic metabolites of loxapine and of amoxapine as well, exhibit antidopaminergic properties. It appears likely that these active metabolites contribute to the clinical effectiveness of loxapine.

Drug metabolites, despite often having similar chemical structure as the parent drugs, may possess different pharmacological effects. The development of fast and reliable analytical methods for the toxicological screening of dibenz[1,4]diazepines and related compounds constitutes a necessary task for laboratories involved in clinical and/or forensic toxicology, but above all for laboratories dealing with the monitoring of the drug metabolites. Indeed, a number of methods have been proposed for this purpose. Most of them are based on high-performance liquid chromatography (HPLC) with spectrophotometric [9–15] or electrochemical detection [16–18].

All substances under consideration possess one or more basic nitrogen groups and are therefore potentially separable by capillary electrophoresis (CE). However, in the literature only one paper was found dealing with the electrophoretic separation and determination of the parent drugs [19], but not taking into account their metabolites.

In the present paper capillary zone electrophoresis (CZE) is introduced for determination based on the adjustment of the electrophoretic mobilities of the protonated solutes, clozapine, loxapine and six metabolites (demethylclozapine, clozapine *N*-oxide, 7-hydroxyloxapine, 8-hydroxyloxapine, amoxapine and 8-hydroxyamoxapine). Beside the variation of the two significant variables pH and ionic strength of the background electrolyte (BGE), a number of additives are applied to work out favorable separation conditions, namely cyclodextrins and a linear soluble polymer, respectively.

The unusual selectivity aspect of the linear, soluble and replaceable polymer polyvinylpyrrolidone (PVP) as pseudo-stationary phase was demonstrated in a number of papers [20–22], including a previous work dealing with similar compounds [23]. PVP can introduce specific interactions with the analytes, which result in a selective reduction of the mobility of the substances, and might lead to an improvement of the resolution.

Cyclodextrins, either native or derivatized, have been introduced to affect the separation selectivity in many cases (cf. e.g., Ref. [24]). In the present investigation, three different cyclodextrins were applied. Two are electrically neutral (β- and γ-cyclodextrin). They differ only in the diameter of their cavity. One cyclodextrin (heptakis-6-sulfato-β-cyclodextrin) is forming a polycharged anion, and migrates therefore in the electric field against the cationic solutes. The interactions of the solutes with the neutral cyclodextrins are based on lipophilic interactions inside the cavity, and hydrophilic interactions with the OH groups at the rim of the molecule. In contrast, additional ion–ion interactions can take place between the oppositely charged separands and heptakis-6-sulfato-β-cyclodextrin (cf. e.g., Ref. [25]).

2. Experimental

2.1. Reagents

The following substances were used as analytes (Fig. 1): clozapine, *N*-demethylclozapine and clozapine *N*-oxide (Novartis Italia, Origgio, Varese, Italy); loxapine, 7-hydroxyloxapine, 8-hydroxyloxapine, amoxapine and 8-hydroxyamoxapine (Lederle Laboratories, Pearl River, NY, USA). Sodium hydroxide (analytical grade), orthophosphoric acid (85%) and γ-cyclodextrin were purchased from E. Merck (Darmstadt, Germany). Polyvinylpyrrolidone (PVP 25) was from Serva (Heidelberg, Germany), β-cyclodextrin from Fluka (Buchs, Switzerland) and heptakis-6-sulfato-β-cyclodextrin from Regis Technologies (Morton Grove, IL, USA). Water was doubly distilled from a quartz apparatus before use.

Stock solutions from pure standard compounds were prepared by dissolving 20 mg of each substances in 20 ml of phosphate buffer, 50 mmol/l, pH 2.5. The working standard solutions were prepared by dilution of stock solutions with double distilled water. All stock solutions were stored at −18°C for

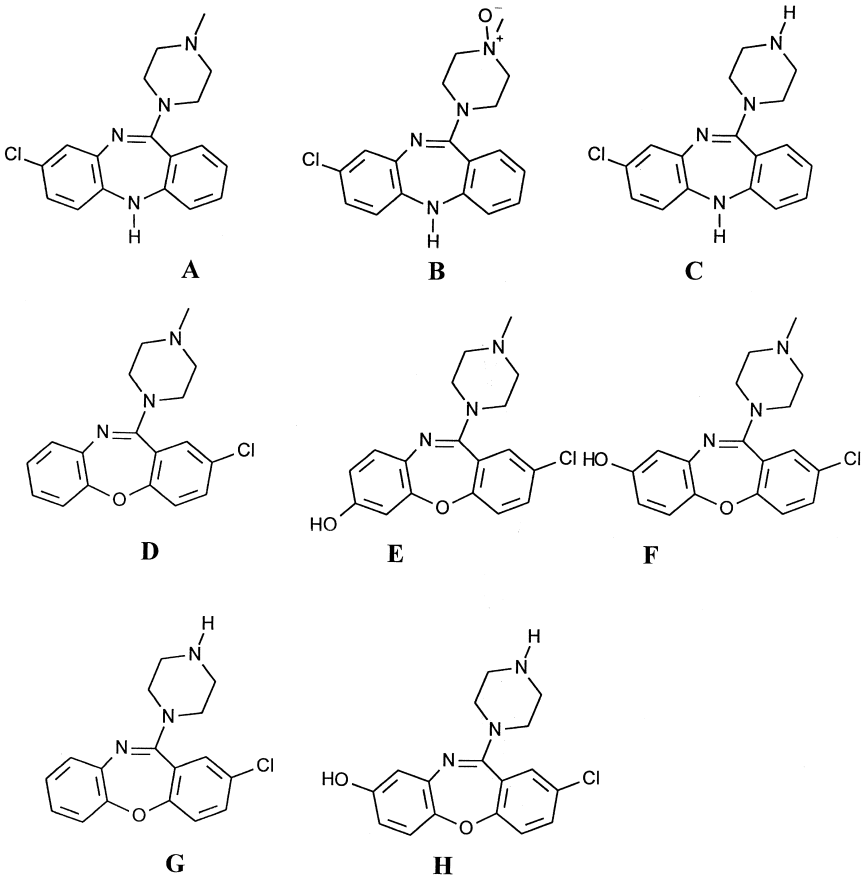

Fig. 1. Structural formulae of clozapine, loxapine and their metabolites. A=Clozapine; B=clozapine *N*-oxide, C=*N*-demethylclozapine; D=loxapine; E=7-hydroxyloxapine; F=8-hydroxyloxapine; G=amoxapine; H=8-hydroxyamoxapine.

one month at most, the standard solutions can be stored at 4°C at most for one week. The CZE buffers were prepared dissolving the suitable volume of phosphoric acid in water and adjusting the pH with a solution of sodium hydroxide (1 mol/l). From this buffer all the others were obtained dissolving the appropriate amount of additive. The buffers were filtered (0.45 μm, Minisart RC25, Sartorius, Göttingen, Germany) prior to use.

2.2. Apparatus

CZE was carried out with a laboratory-made instrument using an uncoated fused-silica capillary (Composite Metal Services, Hallow, UK) of 45.0 cm (effective length 25.2 cm)×50 μm I.D. The sample solutions were loaded into the capillary by electrokinetic injection (2 kV for 5 s, except otherwise stated). The compounds were detected by UV–Vis detection (Spectra System UV 2000, Thermo Separation Products, Riviera Beach, USA). The instrument was operated at 15 kV with currents typically less than 50 μA, generated by a high-voltage power supply (2127 Tachophor, LKB, Bromma, Sweden). The electropherograms were recorded and processed with a dual-channel interface (35900, Hewlett-Packard, Waldbronn, Germany).

2.3. Procedure for capillary preparation and handling

Before use, a new capillary was rinsed for 10 min with deionized water, 10 min with 1 mol/l sodium hydroxide and 15 min with water before filling with

the CZE buffer. Before sample injections the capillary was rinsed with water for 1 min and with CZE buffer for 3 min. For storage overnight, the capillary was additionally washed with water, 0.1 mol/l sodium hydroxide and water.

3. Results and discussion

3.1. Effect of pH and ionic strength of the BGE

Due to the reasons and practical limitations discussed in a previous paper [23] with regard to similar compounds, an acidic pH range of the working buffer is selected here for the separation of the compounds of interest. During the present work it was found that the determination of clozapine *N*-oxide is restricted even to pH lower than 4 (it exhibits very poor reproducibility at higher pH due to decomposition according to literature [26,27]); therefore employment of buffers with pH<4 is necessary.

The variation of the electrophoretic mobility, μ, of the separands in the pH between 2.5 and 3.5 with 5 different buffers (at total phosphate concentration of 50 mmol/l) and plotting μ vs. pH brought the result, that the best separation is achieved at pH 3.5 (plot not shown). However, even at this pH it is not possible to separate loxapine from its metabolite 8-hydroxyamoxapine (D and H in Fig. 1). These two analytes still comigrate.

Variation of the ionic strength of the BGE was carried out in the total phosphate concentration range from 40 to 100 mmol/l (in 10 mmol/l steps). Although no improvement of the resolution for D and H (loxapine and 8-hydroxyamoxapine) was found, the concentration of 60 mmol/l phosphate gave the best peak shape and a time of the analysis less than 5 min. The resulting electropherogram is shown in Fig. 2.

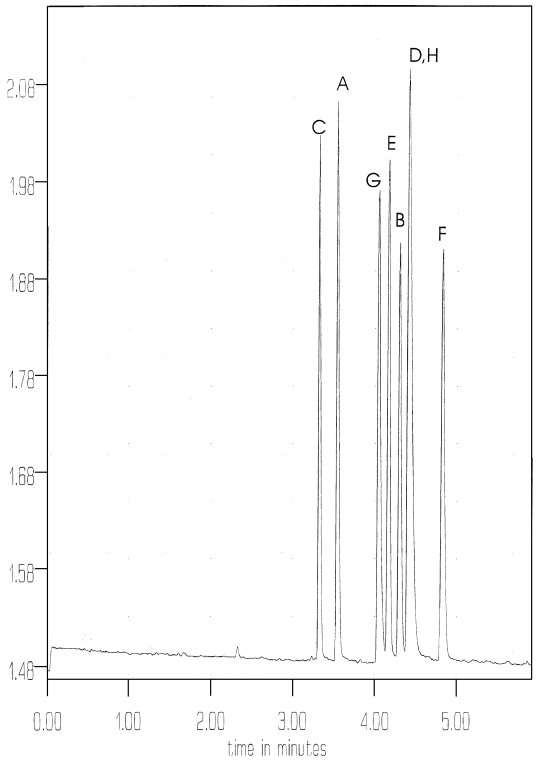

Fig. 2. Electropherogram of the separands with a BGE without additive. Buffer: phosphate, pH 3.5, 60 mmol/l total phosphate concentration. Uncoated capillary: 45.0 cm (effective length 25.2 cm)×50 μm I.D. Detection: UV, 206 nm. Voltage: +15 kV. Analyte concentration: 10 μg/ml. Symbols of the analytes as in Fig. 1.

3.2. Effect of additives

The pH is in many cases that parameter which allows modifying the mobilities of the analytes in a way that a sufficiently high selectivity of separation can be achieved. This mobility is depending on the chemical structure of the analytes, which determines the actual mobility and the p*K* value. However, for some cases these parameters might be so similar for pairs of separands that resolution is not obtained at any pH (and ionic strength). Enantiomeric compounds are well-known examples. Here other strategies to influence the effective mobilities must be applied, e.g., the use of other association equilibria than protolysis. In CE interaction with pseudo-stationary phases like micelles or polymeric additives are used, or agent forming defined complexes with the solutes. As very efficient complex-forming additive cyclodextrins were found, well established for chiral separations, but also possessing high potential to affect selectivity for other separands. It follows from the above results that the variation of pH and ionic strength of the buffer seemingly does not enable to separate all analytes. Consequently such

additives are applied to reach the appropriate resolution.

3.2.1. Effect of polyvinylpyrrolidone used as pseudo-stationary phase

The concentration of PVP in the BGE (phosphate, pH 3.5, 60 mmol/l) was changed in the range between 0 and 0.25% (w/v) (in 0.05% steps). Again mobility vs. concentration PVP plots were derived (not shown). It was found that the best results are achieved using 0.20% PVP. The resulting electropherogram is shown in Fig. 3. It should be mentioned that PVP has an unfavorable UV absorbance at 206 nm; therefore the electropherograms were run here at 245 nm. It can be seen that indeed all eighth compounds are separated. The migration sequence is the same as without additive, but D and H are fully resolved. This improvement must be paid, however, by a small reduction of the resolution of B and E. Note that B and E are metabolites originating not from the same parent drug, but from clozapine or from loxapine, respectively. Therefore for most cases this resolution will be sufficient even for quantitative analysis, because normally only one antipsychotic drug is applied. However, in some rare case resolution of the metabolites from both parent drugs might be demanded.

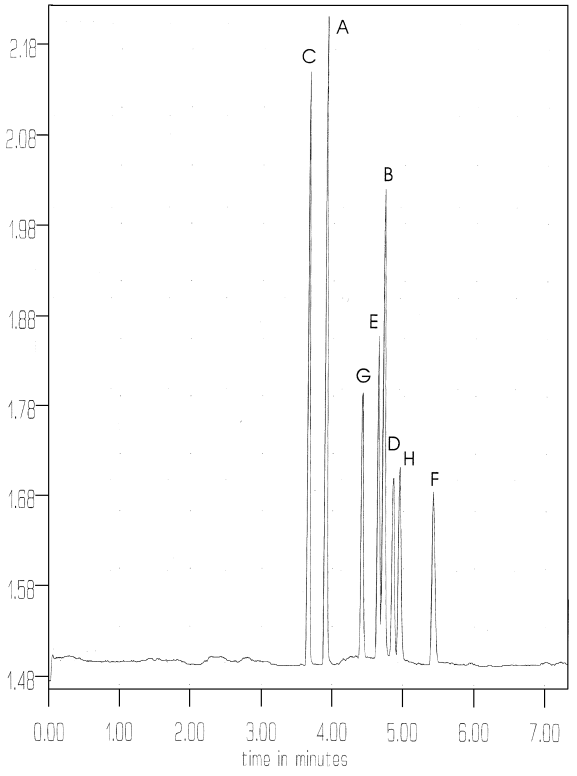

Fig. 3. Electropherogram of the separands with a BGE with 0.2% (w/v) polyvinylpyrrolidone as additive. Buffer: phosphate, pH 3.5, 60 mmol/l total phosphate concentration. Detector wavelength: 254 nm; analyte concentration: 10 μg/ml. Other conditions as in Fig. 2. Symbols of the analytes as in Fig. 1.

3.2.2. Effect of cyclodextrins

3.2.2.1. β-Cyclodextrin

The concentration of β-cyclodextrin was changed in the range between 0.5–4 mmol/l (in six steps). The use of β-cyclodextrin determines the same migration order than PVP. From the dependence of the mobility on the concentration of the additive it could be derived that the most favorable concentration is 0.75 mmol/l. The electropherogram obtained under these conditions is shown in Fig. 4. A similar picture is found in comparison with PVP: in both cases the critical pair is B and E, which is separated, but still no baseline resolution is achieved. As with PVP it follows that the separation performance of this system is sufficient if either loxapine or clozapine have been administered to the patient, because B and E stem from different parent drugs.

3.2.2.2. γ-Cyclodextrin

From the dependence of the mobilities on the γ-cyclodextrin concentration, which was changed between 0 and 5 mmol/l in five steps, the best working conditions for separation were found at 0.50 mmol/l γ-cyclodextrin. As for the additive-free system loxapine and 8-hydroxyamoxapine (D and H) still comigrate. The corresponding electropherogram is shown in Fig. 5. This system is therefore not suitable for the determination of the loxapine metabolites.

3.2.2.3. Heptakis-6-sulfato-β-cyclodextrin

Heptakis-6-sulfato-β-cyclodextrin is a charged additive, which migrates towards the anode, and forms complexes with the positively charged solutes.

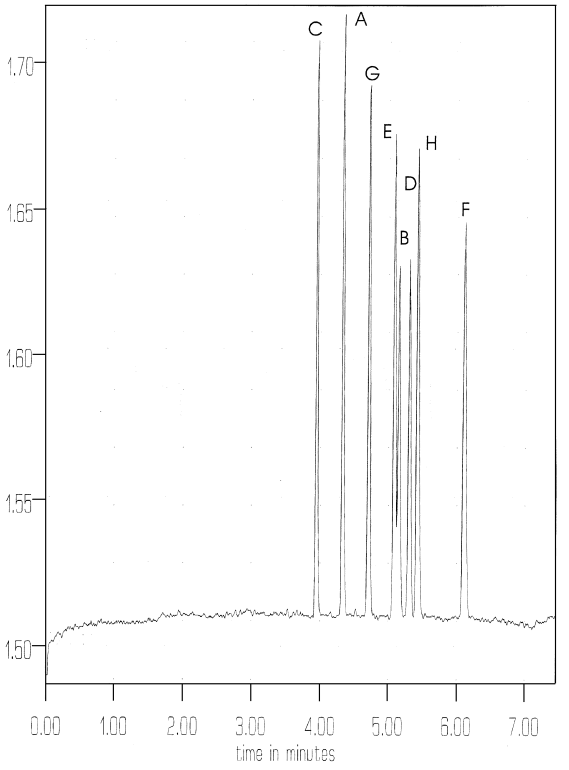

Fig. 4. Electropherogram of the separands with a BGE with 0.75 mmol/l β-cyclodextrin as additive. Buffer: phosphate, pH 3.5, 60 mmol/l total phosphate concentration. Analyte concentration: 1 μg/ml. Other conditions as in Fig. 2. Symbols of the analytes as in Fig. 1.

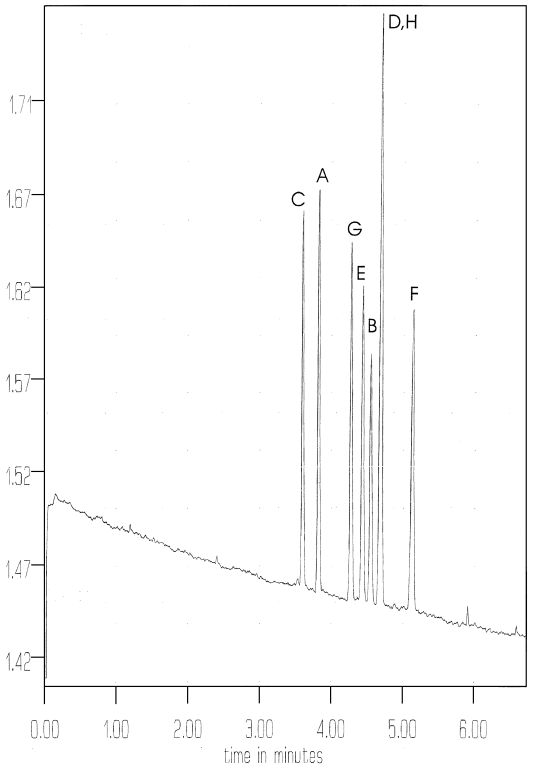

Fig. 5. Electropherogram of the separands with a BGE with 0.50 mmol/l γ-cyclodextrin as additive. Buffer: phosphate, pH 3.5, 60 mmol/l total phosphate concentration. Analyte concentration: 1 μg/ml. Other conditions as in Fig. 2. Symbols of the analytes as in Fig. 1.

The resulting species may have different net charges. Depending on the concentration of the additive (and that of the solute) the association equilibrium results either in a positive net charge (at low concentrations), to a negative net charge (at higher concentration) and even to zero net charge of the species at concentrations in between. Indeed it was found that the separand's complexes are migrating towards the cathode at concentrations of the cyclodextrin of 0.2 and 0.5 mmol/l. The analytes E and D are not fully resolved under these conditions. Increase of cyclodextrin concentration over 0.5 mmol/l leads to a loss of resolution for A and G. The further increase of concentration results in a prolongation of the migration time of the solutes, indicating a reduction of their net positive charge. At 1 mmol/l finally the peaks migrate such slowly that they could not be detected within about 30 min.

The increase of the concentration above 1 mmol/l results in a negative net charge of the complexes, and an increase of the net mobility due to the increase of the charge. Obviously the separands are recorded with reversed order compared to the low concentration conditions. Full resolution of all eight analytes within about 8 min is obtained with 5.0 mmol/l cyclodextrin (Fig. 6). The migration sequence differs compared to those observed for β- and γ-cyclodextrin; especially clozapine *N*-oxide (B) is strongly retarded. A further decrease of migration time by increasing the concentration of the charged cyclodextrin (to concentrations higher than 5 mmol/l) results in an increasing loss of the resolution of compounds F and D. Therefore it can be concluded that the BGE containing 5.0 mmol/l heptakis-6-sulfato-β-cyclodextrin is the most favorable concerning separation and analysis time.

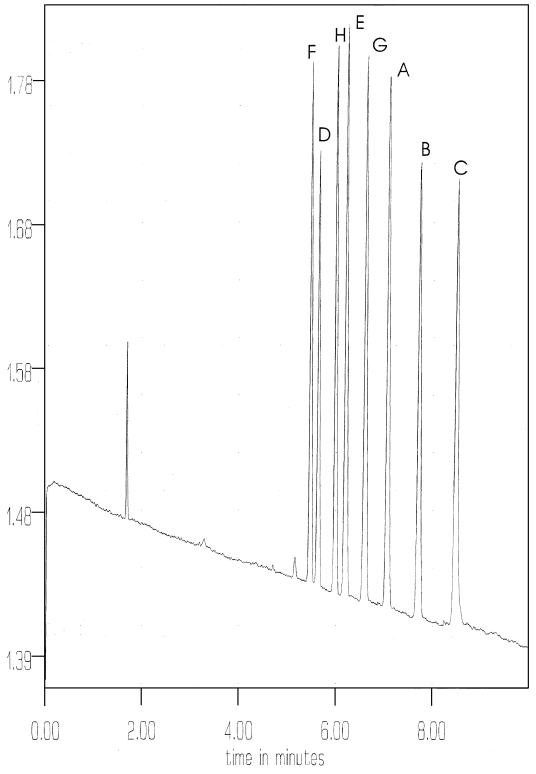

Fig. 6. Electropherogram of the separands with a BGE with 5.0 mmol/l heptakis-6-sulfato-β-cyclodextrin as additive. Buffer: phosphate, pH 3.5, 60 mmol/l total phosphate concentration. Voltage: −15 kV; sample was loaded into the capillary here by siphoning (height difference of the reservoirs of 10 cm for 10 s). Analyte concentration: 10 μg/ml. Other conditions as in Fig. 2. Symbols of the analytes as in Fig. 1.

4. Conclusions

(i) The variation of the pH (within 2.5 and 3.5) and the ionic strength does not enable the separation of loxapine from all metabolites.

(ii) γ-Cyclodextrin as additive of the BGE does not lead to an improvement of the separation of the critical pair of analytes compared to the system without additive.

(iii) PVP and β-cyclodextrin as additives enable the separation of all metabolites from the same parent drug. If both antipsychotic drugs have been administered, the clozapine metabolite B, and the loxapine metabolite E, although separated, did not show baseline resolution.

(iv) Due to its combined interaction with the separands (lipophilic, hydrophilic, ion–ion interactions) the application of heptakis-6-sulfato-β-cyclodextrin as additive at moderate concentration enables full resolution of all eight compounds within 8 min. If there is need to determine all metabolites from both parent drugs, these conditions are the most favorable.

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