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Journal of Chromatography B, 708 (1998) 249–256

JOURNAL OF CHROMATOGRAPHY B

Determination of flufenamic, meclofenamic and mefenamic acids by capillary electrophoresis using β-cyclodextrin

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Received 30 September 1997; received in revised form 11 December 1997; accepted 16 December 1997

Abstract

The possibility of separating flufenamic, meclofenamic and mefenamic acids by capillary electrophoresis was studied. The best approach involved combining a suitable pH of the carrier electrolyte (pH 12.0) with the host–guest complexation effects of β-cyclodextrin. A running buffer consisting of 30 m*M* phosphate buffer (pH 12.0), 2 m*M* β-CD and 10% (v/v) acetonitrile was found to provide a very efficient and stable electrophoresis system for the analysis of fenamic acids by capillary zone electrophoresis. Responses were linear from 0.4 to 40 μg/ml for the three drugs with detection limits of about 0.3 ng/ml. Intra- and inter-day precision values of about 1–2% R.S.D. ($n=11$) and 3–4% R.S.D. ($n=30$), respectively, were obtained. The method is highly robust and no breakdowns of the current or capillary blockings were observed for several weeks. The general applicability of this rapid CZE procedure (migration times less than 12 min) is demonstrated for several practical samples, including serum, urine and pharmaceuticals.

Keywords: Flufenamic acid; Meclofenamic acid; Mefenamic acid

1. Introduction

Flufenamic acid (FFA: *N*-(α,α,α-trifluoro-*m*-tolyl)anthranilic acid), meclofenamic acid (MCFA: *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid) and mefenamic acid (MFA: *N*-(2,3-xylyl)anthranilic acid) belong to a family of nonsteroidal antiinflammatory drugs (NSAIDs) that are derivatives of *N*-phenylanthranilic acid. They are used as potent analgesic and antiinflammatory agents in the treatment of osteoarthritis, rheumatoid arthritis and other painful musculoskeletal illnesses. The fenamates appear to owe these properties primarily to their capacity to inhibit cyclooxygenase. Unlike other NSAIDs, they may also antagonize certain effects of prostaglandins [1].

Several methods have been recommended for the individual determination of fenamic acids. These depend on titrimetric [2], spectrophotometric [3–5], fluorometric [6,7] and flow-injection [8] techniques. Gas–liquid chromatography has been proposed for the determination of MFA in human serum [9] and FFA in rat plasma and uterus [10]. Liquid chromatography has also been used for the determination of MFA [11]. However, no reports were found concerning the simultaneous determination of these drugs.

Capillary electrophoresis (CE) is a highly efficient and rapid technique for the separation and quantitative analysis of a wide range of charged and, to a lesser extent, uncharged species [12,13]. The tech-

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0378-4347/98/$19.00
PII S0378-4347(97)00674-9

nique has been extensively used for the separation of inorganic and organic anions.

Cyclodextrins (CDs) are by far the most common of all chiral additives used in liquid chromatography and CE. Other applications of CDs based on their inclusion complexation ability with a large number of aromatic ring-containing compounds have yet to receive adequate attention. Enhancement of selectivity by the use of CDs is usually attributable to their ability to selectively include a wide variety of guest organic and inorganic molecules or ions into their hydrophobic cavity.

The use of β-CD in capillary zone electrophoresis (CZE) to decrease analysis times and improve reproducibility during the determination of food colourants has been recently reported [14,15].

The purpose of this work was to investigate the separation of FFA, MCFA and MFA by CE. The pH of the carrier electrolyte, the presence of detergents and host–guest complexation using β-CD were the three routes studied. The last approach, where the separation of fenamic acids is carried out via differentiating complexation of β-CD, gave the best resolution and the shortest analysis time.

The proposed CZE method is simple, reliable and very useful for therapeutic drug monitoring in hospitals because of the ability of automation and short analysis time. The method was use to determine FFA, MCFA and MFA in pharmaceutical formulations and serum and urine samples.

2. Experimental

2.1. Apparatus and running conditions

A capillary electrophoresis P/ACE System 5500 (Beckman Instruments, Palo Alto, CA, USA) equipped with a diode-array detector, an automatic injector, a fluid-cooled cartridge and a System Gold data station were used in this study. Electrophoresis was performed in a 57 cm×75 μm I.D. (50 cm to the detector) fused-silica capillary tube (Beckman Instruments). The high voltage power supply was set to 12 kV (normal polarity, equivalent to a field strength of 210 V/cm) resulting in a typical current of 90–92 μA. Sample introduction was made at the anode side using the pressure option (0.5 p.s.i.≈33.3 mbar) for 4–10 s. Before the first use, the capillary was conditioned by flushing for 5 min with freshly prepared 0.1 *M* sodium hydroxide and rising with the buffer. It was then equilibrated with the buffer for 15 min applying the separation voltage of 12 kV. Between experiments, the capillary was rinsed with buffer for 2 min. If the species or the concentration of the additive was changed, the capillary was first rinsed with 0.1 *M* sodium hydroxide followed by the new buffer. The capillary inlet and outlet vials were replenished after every ten injections. When long automated sequences of samples were being run, different wash and run buffer vials were used to avoid lowering the fluid level in the run buffer vial and consequent hydrodynamic siphoning. If not otherwise stated, a 30 m*M* phosphate buffer of about pH 3.0 containing β-CD (2 m*M*) and acetonitrile (10% v/v) was used as the running buffer.

Absorbance spectra of FFA, MCFA and MFA dissolved in the electrophoretic buffers were recorded with a Unicam UV/Vis spectrophotometer (Unicam Limited, Cambridge, United Kingdom) with a 1-cm path-length quartz cell to identify the peaks obtained. The detection of analytes was performed at 285 nm.

The pH of the electrophoretic buffers was adjusted by means of a model pHM 62 Standard pH-meter from Radiometer (Copenhagen, Denmark).

2.2. Reagents and solutions

The chemicals used for preparation of the solutions were purchased at the highest grade possible. Demineralized water from a Milli-Q system (Millipore Iberica, Madrid, Spain) was used for preparing the solutions. Phosphoric acid, hydrochloric acid, sodium dihydrogen phosphate, sodium tetraborate and sodium hydroxide were of analytical-reagent grade, and are available from Merck (Darmstadt, Germany). Methanol, ethanol and acetonitrile (HPLC grade) were obtained from Romil (Loughborough, Leics, UK). Flufenamic, meclofenamic and mefenamic acids, α-, β- and γ-cyclodextrins, sodium dodecyl sulphate, cholic acid (sodium salt) and taurocholic acid (sodium salt) were purchased from Sigma (St. Louis, MO, USA).

Stock standard solution of FFA, MCFA and MFA were prepared by dissolving the required amount of

the corresponding compound (Sigma) in 0.01 *M* sodium hydroxide and diluting with demineralized water up to 250 ml in a calibrated flask. These solutions showed a pH of about 8.5 and remained stable for a least two weeks if kept refrigerated. Working solutions of lower concentrations were freshly prepared by dilution of the stock solution with demineralized water.

For the studies where a modifier was added to the electrophoretic solution, the buffer solution was mixed with the required amount of the modifier and then the pH was readjusted with 0.02 *M* hydrochloric acid or sodium hydroxide when necessary.

All solutions were filtered through a 0.45 μm filter (Millisolve Kit, Millipore), and then degassed by sonication and evacuation.

3. Results and discussion

Fenamic acids precipitate under acidic conditions; therefore, measurements were always carried out at pH values higher than 8.0. Because the electroosmotic migration velocity is greater than the electrophoretic migration velocity of the acids tested, a positive potential should be applied across the capillary and the acids should migrate from the source to the detector at a velocity lower than that of the electroosmotic flow.

In order to find separating conditions that provide a complete resolution of FFA, MCFA and MFA in one CE run, three approaches were investigated: (1) CZE separation based on differences in the migration times of the fenamic acids originating in the p*K* values and the mobilities of their anions; (2) MEKC separation based on differences with the partition of the fenamic acids and/or their ionic forms between appropriate micelles and running buffers; (3) CZE separation using differences in the effective mobilities of the ionic form of the fenamic acids in the presence of CDs.

3.1. CZE separation at various pH values

The separation of FFA, MCFA and MFA was investigated using phosphate and/or borate buffer. The peaks were easily confirmed by comparison of the absorption spectra obtained from electropherogram with those of the standards in the same buffer solution.

The dependence of the migration times of fenamic acids on the pH of the carrier electrolyte is presented in Table 1. We can see that at pH 12 FFA can be separated from MCFA or MFA, although the last two drugs failed to resolve. Considering a relationship between the effective mobility of each acid, its p*K* value, actual mobility of its ionic form and pH of the carrier electrolyte [12], it is clear that the three fenamic acids cannot be separated effectively.

The addition of organic solvents to the electrophoretic buffer was also considered, although this effect is hard to predict because it affects several variables, including viscosity, dielectric constant and zeta potential. The presence of acetonitrile, methanol or ethanol in the electrophoretic electrolyte lowered the current and slightly improved the separation at the expense of a longer analysis time. It is of interest to stress that acetonitrile yielded shorter migration times than methanol and ethanol and the base line was more stable; therefore, a 10% (v/v) acetonitrile was added to electrophoretic electrolyte in all subsequent studies.

3.2. Micellar electrokinetic capillary chromatography

Analytes are now mainly separated by observing the difference in partitioning between the micellar phase and the aqueous phase and also according to electrophoretic mobility. This technique has been reported to have advantages over CZE in the separation of both electrically neutral and ionic substances [12,13].

There are several ways of manipulating the selec-

Table 1
Comparison of migration times (min) of FFA, MCFA and MFA at different pHs

Compound	pH				
	9.8	10.6	11.0	11.6	12.0
FFA	5.67	5.86	5.78	6.83	6.87
MCFA	5.67	5.86	5.78	6.91	6.99
MFA	5.67	5.86	5.78	6.93	7.02

Experimental condition: 15 kV, and 20 m*M* sodium phosphate carrier solution. A fused-silica capillary of 57 cm×0.075 mm I.D. (50 cm to detector) was used

tivity in MEKC, the most effective of which is to change the type of surfactant [16]. In this respect, a method was developed in which anionic, cationic and nonionic surfactants [sodium dodecylsulphate (SDS), 10 m*M*; sodium cholate (SC), 10 m*M*; sodium taurocholate (STC), 10 m*M*; cetyltrimethylammonium bromide (CTAB), 10 m*M*; and triton X-100 (0.4% v/v)] in 20 m*M* phosphate buffer (pH 12.0) containing 10% (v/v) acetonitrile were studied.

Triton X-100, SC and STC were not suitable as micelle modifiers because MCFA and MFA could not be separated. CTAB was also unsuitable because the migration times were very long (more than 30 min) and MCFA eluted together with MFA. When SDS was added to the running electrolyte, a clear improvement of the separation was observed and all three analytes showed longer migration times with increasing SDS concentration. The resolution of the peaks corresponding to FFA/MCFA and MCFA/MFA as a function of SDS concentration is shown in the Fig. 1. As can be seen, an SDS concentration of 5 m*M* is the best compromise for achieving a good separation of three fenamic acids.

Fig. 2 shows an electropherogram at 285 nm and absorbance spectra obtained using an electrophoretic solution containing 20 m*M* phosphate buffer (pH 12.0), 5 m*M* SDS and 10% (v/v) acetonitrile. The fenamic acids were separated within 10 min with good reproducibility although the resolution of the peaks corresponding to MCFA and MFA was not very good. The relative standard deviations of the migration times and the peak areas were in the ranges 0.1–0.6% and 1.3–2.1%, respectively ($n=6$). Using peak area, the calibration graphs were linear up to 30 μg/ml.

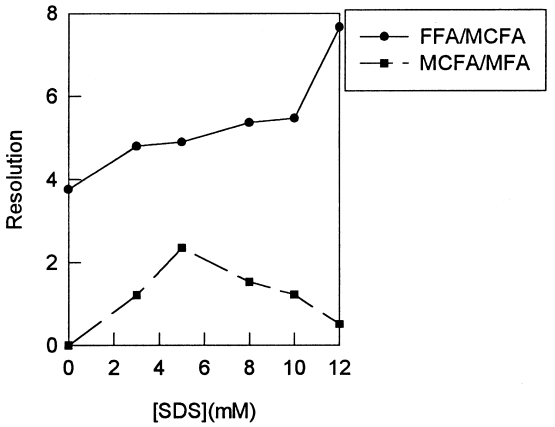

Fig. 1. Influence of SDS concentration on the separation of fenamic acids. Background electrolyte=20 m*M* phosphate buffer (pH 12.0) containing 10% acetonitrile.

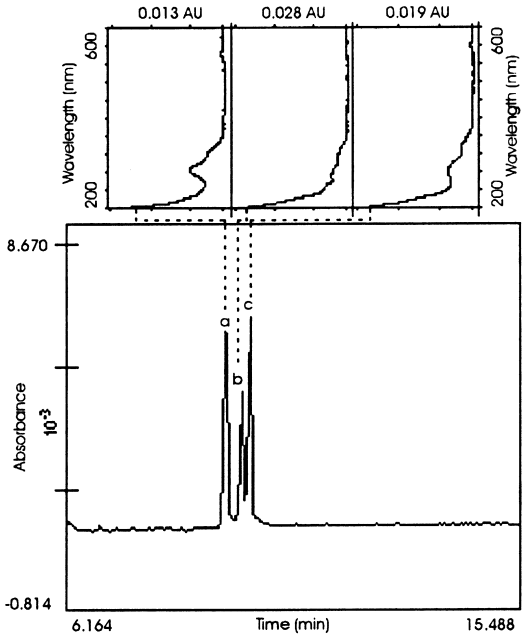

Fig. 2. Electropherogram at 285 nm and the absorbance spectra of three fenamics acids. Peak identification (a) FFA, (b) MCFA and (c) MFA. The separation was driven by an electrolyte solution containing 20 m*M* phosphate buffer (pH 12.0), 5 m*M* SDS and 10% (v/v) acetonitrile. Voltage applied 15 kV.

3.3. CZE separation via host–guest complexation

Electrophoretic mobility depends on charge density, and so any manipulation of the molecular mass of a substance can affect the mobility and consequently the migration time. In order to achieve a better separation of the three fenamic acids, we investigated the possibilities of improving the separation by addition of CDs to the working electrolyte.

CDs are macrocycles formed with 6, 7 or 8 glucopyranose units and are termed α-, β- or γ-, respectively. The hydrophobic internal cavity permits the formation of inclusion complexes with analytes that are capable of conforming to their interior size dimensions. The relative stabilities of these inclusion compounds may be influenced by factors such as

hydrogen bonding, hydrophobic interactions, solvent effects and size and shape of the analytes. β-CD with a cavity diameter of 6.0–6.5 Å [12] has proved to be the most useful for the separation of the fenamic acids.

Owing to the formation of inclusion complexes between the ionic forms of fenamic acids and β-CD, which have higher molecular masses than free anions, the migration time decrease in all cases. The dependence of the effective mobilities of FFA, MCFA and MFA on the concentration of β-CD in the carrier electrolyte is plotted in Fig. 3. These plots shows that this complexing agent affected the migration of the fenamic acids in a different manner. The stability constants and the ionic mobilities of the fenamates and their complexes are responsible for this effect. When the concentration of β-CD in the carrier electrolyte increased, the migration time of each fenamic acid decreased with varying patterns. The optimum resolution was achieved using a β-CD of 2 m*M*.

The effect of electrolyte concentration on the mobility of FFA, MCFA and MFA in the presence of β-CD was studied by increasing the phosphate buffer (pH 12.0) concentration from 5 to 40 m*M* in the electrophoretic solution. An increase in the ionic strength of the buffer improved the resolution and increased the migration time of the three drugs. To limit the Joule heat generated inside the capillary, the maximum voltages were chosen from the Ohm's law plot. The best separation was achieved with a voltage of 12 kV and using a buffer concentration of 30 m*M*.

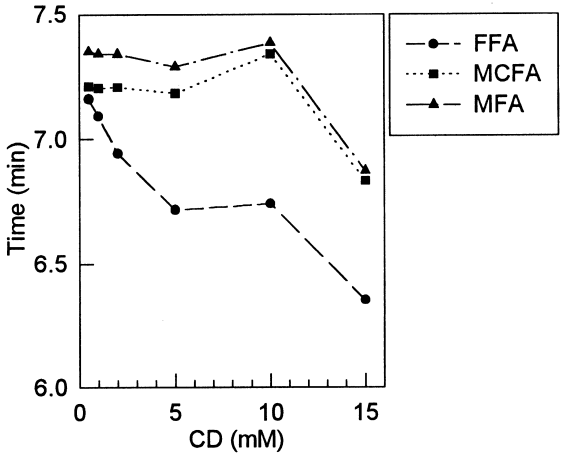

Fig. 3. Effect of β-CD on migration times of fenamics acids. β=CD was added to 20 m*M* sodium phosphate buffer (pH 12.0) containing 10% (v/v) acetonitrile.

As the temperature was raised (18–35°C), the decrease in viscosity of the running electrolyte and the increasing current inside the capillary shortened the migration time. To obtain reproducible results it is very important to keep the temperature constant during the analysis by using capillary cooling. The separation was carried out at 25°C.

A study was performed to determine the degree of trace enrichment at various injection times and any sacrifice in resolution that resulted. It was possible to perform pressure injection up to 20 s without substantial loss in resolution and with fair linearity of peak area and height with injection time (analyte quantity).

Summarizing, the optimum conditions for the separation of FFA, MCFA and MFA are: potential applied across the capillary, 12 kV; capillary temperature, 25°C; running buffer, 2 m*M* β-CD in 30 m*M* phosphate buffer (pH 12.0) containing 10% (v/v) acetonitrile; hydrodynamic sample introduction, 0.50 p.s.i. for 4–10 s.

3.3.1. Analytical performance characteristics

Under the optimum conditions above described, the samples can be analysed quantitatively. Table 2 gives the results of their statistical evaluation. The calibration graphs showed good linearity over the range 0.4–40 μg/ml. The migration of the fenamic acids was very reproducible, R.S.D. within the range 0.3–0.5%. The detection limits were estimated through the IUPAC model conveniently modified for chromatography [17]. The values summarized in Table 2 show the high sensitivity of the method.

Reproducibilities in the peak-area measurements were studied by injecting eleven replicate solutions containing 2 μg/ml of each analyte. The peaks corresponding to the fenamic acids (see Fig. 4) are completely separated and the responses for FFA, MCFA and MFA were constant for eleven consecutive runs with a R.S.D. of 1.1%, 1.4% and 1.9%, respectively, for each peak area. When the same sample was run 30 times over a five day period, the R.S.D. were 2.8%, 3.4% and 4.1% for FFA, MCFA and MFA, respectively.

Table 2
Analytical data for the CZE determination of the fenamic acids (peak area, arbitrary units, versus, concentration μg/ml)

Compound	*B*	*A*	r^2	D.L. (ng/ml)	S.E.	*N*
FFA	0.0707±0.0008	0.04±0.01	0.9994	0.308	180 000	9
MCFA	0.0343±0.0005	0.04±0.01	0.9995	0.523	200 000	8
MFA	0.0660±0.0009	0.09±0.02	0.9992	0.363	190 000	9

B is the slope of the regression lines fitted to the calibration data set±standard deviation; *A* is the intercept of the regression lines fitted to the calibration data set±standard deviation; r^2 is the correlation coefficient of the calibration graph; $S_{y.x}$ the standard deviation from the calibration graph; D.L. is the detection limit (signal-to-noise ratio=3); S.E. is the separation efficiency (number of theoretical plates per meter); and *N* is the number of the experimental data points.

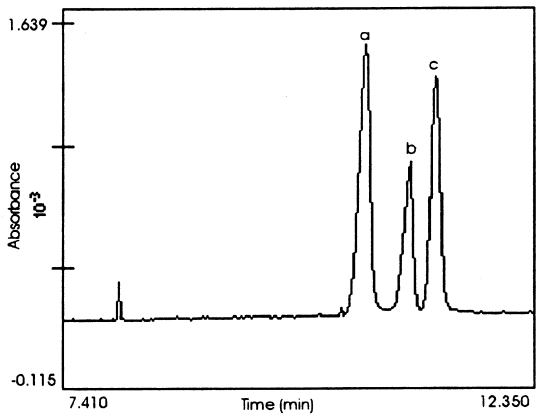

Fig. 4. CZE separation of fenamic acids. Peak: a=FFA; b=MCFA; c=MFA. Running buffer: 30 m*M* phosphate (pH 12.0), 2 m*M* β-CD, 10% (v/v) acetonitrile.

3.3.2. Applications

The CZE assay in the presence of β-CD is characterized by long-term stability and reproducibility. More than 3000 analyses could be performed without replacement of the capillary. To demonstrate the usefulness of the procedure for the determination of fenamic acids, pharmaceutical formulations and human serum and urine were analysed for the presence of these drugs.

Analysis of drug in urine samples by CZE is always a delicate problem. If urine is directly injected into the capillary, proteins and the other biomolecules in the urine matrix are adsorbed to the wall of the capillary and thus quickly harm the column's performance. In addition, appropriate conditions must be found where no other components comigrate with the analytes.

Experiments with different urine dilution ratios have shown that a 10-fold dilution of urine was suitable for the analysis because of FFA, MCFA and MFA were kept free of adverse matrix effects.

Known amounts of the analytes (between 3 and 20 μg/ml) were spiked in ten times diluted urine to establish the calibration curve. In the concentration ranges studied the calibration curves were linear and the migration times were reproducible (R.S.D. less than 0.6% were always obtained). Table 3 summarizes the results obtained and Fig. 5 shows the

Table 3
Recoveries of fenamic acids in real samples

Sample	FFA			MCFA			MFA		
	Added[a] (ppm)	Mean recovery (%)	R.S.D. (%)	Added (ppm)	Mean recovery (%)	R.S.D. (%)	Added (ppm)	Mean recovery (%)	R.S.D. (%)
Urine 1	3.0 (5)	101.1	1.6	3.0 (5)	95.0	1.6	3.0 (5)	99.9	2.0
Urine 2	10.0 (6)	98.0	2.81	0.0 (6)	97.9	2.9	10.0 (6)	98.6	2.2
Urine 3	20.0 (6)	99.8	1.82	0.0 (6)	101.6	1.9	20.0 (6)	102.1	1.7
Serum 1	3.0 (4)	94.8	2.7	3.0 (4)	102.2	2.4	3.0 (4)	102.7	2.7
Serum 2	12.0 (5)	94.0	1.0	12.0 (5)	101.4	1.2	12.0 (5)	98.9	1.4
Serum 3	30.0 (5)	98.5	2.4	30.0 (5)	95.7	0.7	30.0 (5)	98.0	1.2

[a] Number of samples is in parenthesis.

Table 4
Determination of FFA, MCFA and MFA in real pharmaceutical formulations

Preparation	Formulation	Drug	Theoretical amount	Experimental amount[a] (mg)	Purity (%)
Movilisin (Luitpold)	Spray	FFA	30 mg/ml	29.4±2.6	98.0
Movilisin (Luitpold)	Gel	FFA	30 mg/g	29.3±1.0	97.6
Movilisin (Luitpold)	Cream	FFA	30 mg/g	29.7±0.6	99.0
Meclomen (Parke-Davis)	Capsule	MCFA	113.5 mg	109.6±1.6	96.6
Coslan (Parke-Davis)	Suppository	MFA	250 mg	248.4±1.6	99.4

[a] Means of four samples±standard deviation.

separation of the fenamic acids under the best conditions for a human urine sample.

The serum samples were spiked with different quantities of these drugs, so that their concentrations were similar to those used in clinical applications [1]. The serum was treated with acetonitrile to separate the proteins [18]. After centrifugation (3 min at 1000 *g*), the liquid supernatant was treated with 0.1 ml of 0.01 *M* sodium hydroxide, filtered through a 0.45-μm filter and diluted with demineralized water to a appropriate volume. In the concentration range studied (3–30 μg/ml for each drug), the calibration curves were linear and the migration times were reproducible. The results obtained in the analysis of three different samples are indicated in Table 3.

The method developed was also used to quantify medicaments and the results obtained can be see in Table 4.

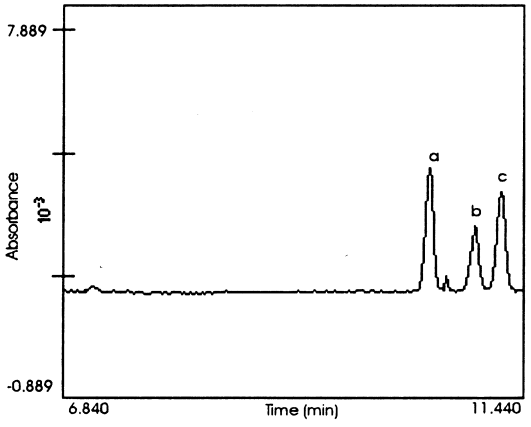

Fig. 5. Electropherogram of urine-1 sample. Hydrodynamic injection (6 sg). Other conditions as for Fig. 4.

4. Conclusions

Host–guest complexation of the ionic form of the fenamic acids (fenamates) with β-CD yields advantages for the simultaneous determination of these compounds in CZE. The complexation with β-CD decreases the charge density and the electrophoretic mobility of fenamates. Consequently, they eluted faster and migration times decreased according to the ability of each analyte to form complexes.

Considering the results related to the quantitations (reproducibilities in peak-area), short (<12 min) and reproducible migration times, it is apparent that the proposed CZE method is a very promising alternative to the determination of fenamic acids. In addition, the data obtained in the analysis of FFA, MCFA and MFA in human serum and urine samples and in pharmaceutical formulations demonstrate the applicability of the method.

Acknowledgements

The authors would like to acknowledge the financial of DGICYT (Ministerio de Educación y Ciencia de España) (Project No. PB96-1101) and Comunidad de Murcia.

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