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Separation of fibrate-type antihyperlipidemic drugs by capillary electrophoresis and their quantitation in pharmaceuticals

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Abstract

Six antihyperlipidemic agents—bezafibrate, ciprofibrate, clofibrate, clofibric acid, fenofibrate and gemfibrozil were separated by means of capillary electrophoresis, using unmodified fused silica tubing of 75 μm internal diameter and 87 cm length (65 cm to the UV detector at 227 nm). Migration time and selectivity were examined in differing pH of separation buffer, varying separation voltage and differing temperature. Optimal separation was achieved using 1/15 M phosphate buffer pH 10, 240 V/cm at 25 °C. The optimal separation conditions were then used to elaborate the method of quantitation of bezafibrate, ciprofibrate and gemfibrozil in Bezamidin®, Lipanor® and Gemfibral® pharmaceuticals. The clofibric acid was used as internal standard. The calibration curve was constructed from 0.2 to 0.8 mg/ml of each compound and 0.5 mg/ml of internal standard. The calibration data were proved to be linear by Mandel and Lack-of-fit tests. Statistical evaluation of results proved proper recovery of elaborated method (102.42, 97.32 and 101.51%, respectively) and good repeatability (9.51, 5.52 and 11.15%, respectively). The linearity of recovery was also tested by analyzing increasing amount of the samples. Three fortified samples of each drug were also analyzed to perform additional accuracy validation.

Keywords: Bezafibrate; Ciprofibrate; Clofibrate; Clofibric acid; Fenofibrate; Gemfibrozil; Capillary electrophoresis

1. Introduction

Fibrates are a group of antihyperlipidemic agents, widely used in current treatment of different forms of hyperlipidemia and hypercholesterolemia. They are 2-phenoxy-2-methylpropanoic acid derivatives. The group includes bezafibrate (2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoic acid), ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropanoic acid), clofibrate (2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester), clofibric acid (2-(4-chlorophenoxy)-2-methylpropanoic acid), fenofibrate (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid 1-methylethyl ester), and gemfibrozil (5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid).

Most of the papers regarding quantitation of fibrates concern their determination in biological material. Only a few papers present their quantitation in pharmaceutical formulations. Spectrophotometric determination of clofibrate in pharmaceuticals, based on reaction with hydroxylamine, was described by Agrawal and Patel [1] and Shaii et al. [2]. The purple complex of the derivative formed with iron was quantified at 530 nm. The direct spectrophotometric determination of clofibrate in gelatinous capsules was described by Aftalion et al. [3] who compared several methods of extraction and quantified the substance at 230, 265 and 280 nm.

The HPLC quantitation in pharmaceuticals was also performed for clofibrate, bezafibrate and gemfibrozil. Bellotti and Frigo [4] determined clofibrate in capsules, using LiChrosorb® C8 column and mobile phase containing acetonitrile and water in different proportions. Ejima et al. [5] quantified also clofibrate in capsules. He used Zorbax® CN column and mobile phases: acetonitrile–60% phosphoric acid (1000:1 v/v, one volume of 60% phosphoric acid) and hexane–propan-2-ol–acetic acid (98.45:1.5:0.05 v/v/v). Shafiee and Shojaie [6] performed quantitation of clofibrate on Bondapak® C18 column. In all cases, UV detection was used.

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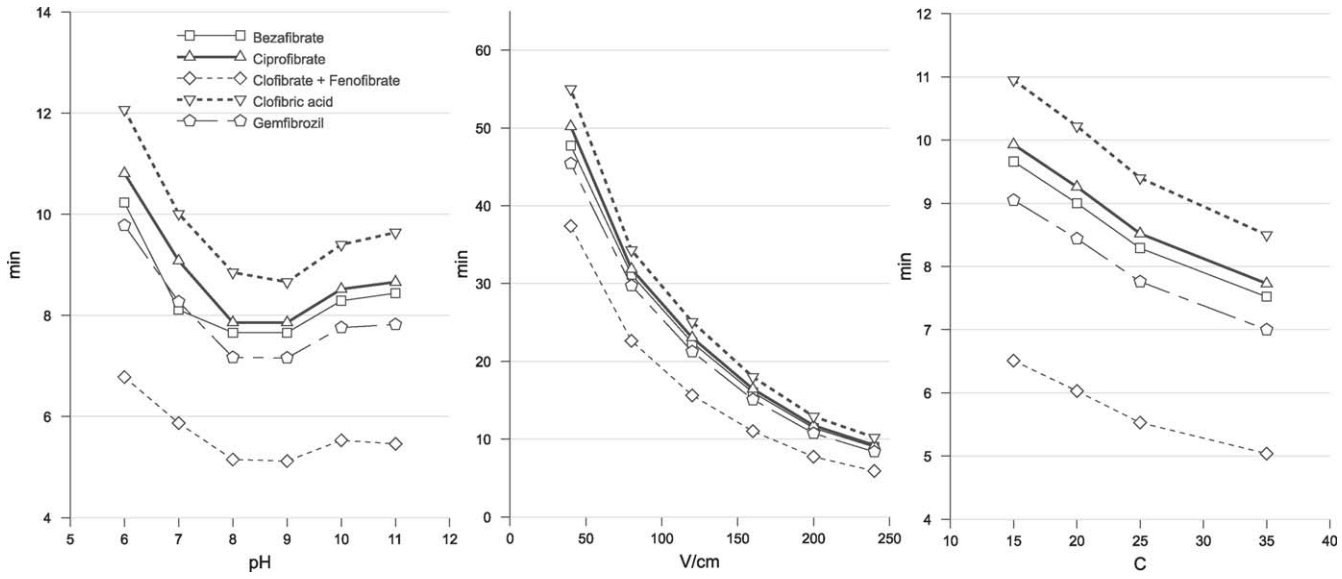

Fig. 1. Migration time of fibrates vs. pH of separation buffer (240 V/cm), separation voltage at pH 10 and temperature at pH 10 and 240 V/cm.

Only two papers describe HPLC determination of the other drugs. Bezafibrate was quantified by Zarapkar et al. [7]. on Bondapak® C18 column, using methanol–water–acetic acid (80:20:1 v/v/v) as mobile phase, and UV 230 nm detection. Li et al. [8] performed quantitation of gemfibrozil in pharmaceuticals on C18 column with mobile phase methanol–water–acetic acid (75:24:1 v/v/v) and UV 276 nm detection.

The papers concerning capillary electrophoresis (CE) are very sparse. Ahrer and Buchberger [9] performed separation of drugs found in river sediments (bezafibrate and clofibric acid among others) by HPLC and CE. The liquid–liquid and SPE were used to prepare samples for CE. They used acetate buffer and MS/MS detection. In another review paper [10], the same authors reported possibility to detect drugs (and fibrates among them) in ng/l concentration. Third article, written by Ahrer et al. [11] describes similar method for determination of bezafibrate and clofibric acid (among others) in surface water.

Vincent and Vigh [12] adapted non-aqueous CE to separate chiral isomers of different drugs, with ciprofibrate among them. They used heptakis(2,3-diacethyl-6-sulphate)-β-cyclodextrin as the chiral separation agent.

The only other work regarding biomedical application of CE fibrates determination was published by Hütterman and Blaschke [13]. They have determined ciprofibrate and its glucuronide in human urine, with and without enantioselective separation. Unmodified fused silica tubing was used. Achiral separation was made using borate buffer, chiral separation was performed with phosphate buffer with β-cyclodextrin addition.

There are no papers regarding separation of fibrates, nor quantitation in pharmaceutical formulations by CE. Thus, we have decided to elaborate such method and perform its validation, as continuation of our earlier work on quantitation of currently used fibrates in pharmaceuticals: HPLC [16], densitometry and videodensitometry [17,18], and derivative spectrophotometry [19].

2. Experimental

2.1. Chemicals and samples

Bezafibrate, clofibrate, clofibric acid, fenofibrate and gemfibrozil were purchased from Sigma (St. Louis, USA). Ciprofibrate was obtained from Sanofi-Synthelabo (France). Bezamidin® tablets (200 mg of bezafibrate, Pliva, Cracow, Poland) and Gemfibral® tablets (450 mg of gemfibrozil, Polpharma, Poland) were purchased in local drugstore. Lipanor® capsules (100 mg of ciprofibrate, Sanofi-Synthelabo, Warsaw, Poland) were obtained from manufacturer. Analytical grade methanol was used to obtain solutions for analysis (1 mg/ml). The salts used to prepare phosphate buffer (KH_2PO_4 and Na_2HPO_4) were of "Ultrapure Bioreagent"® (JTBaker, UK) grade. Fresh double distilled water was used for buffer solution preparation.

2.2. Instrumentation

The PrinCE CE kit with UV Lambda 1010 detector at 227 nm was used. Drugs were separated on unmodified 75 μm silica tubing of 87 cm length (65 cm to the detector). The coating on the capillary was partially removed by burning at the point of detection, and this part was assigned onto the detection block. Capillary was conditioned by 1 M NaOH before use, and by 0.1 M NaOH (2 min, 2000 mbar pressure) before each run. The separation was performed using 1/15 M phosphate buffer of pH 6–11. The sample was spiked at 20 mbar pressure, during 5 s, at anionic end. The separation voltage was in range of 40–240 V/cm.

2.3. Separation optimization

For optimization of electrophoretic separation, we prepared methanolic 1 mg/ml stock solutions of each drug. The solutions were analyzed by means of CE, examining dependence of mi-

gration time and selectivity on the following conditions (Fig. 1):

(1) pH of the separation buffer in range 6–11,
(2) separation voltage in range 40–240 V/cm,
(3) temperature in range 15–35°C.

2.4. Calibration procedure

Stock standard solutions with concentration 2 mg/ml of bezafibrate, ciprofibrate and gemfibrozil were prepared in methanol. Calibration solutions containing 0.2–0.8 mg/ml were obtained by appropriate dilution of the stock solution with the same solvent. Internal standard (clofibric acid) was added to obtain its 0.5 mg/ml concentration in all cases.

2.5. Quantitative assay

After optimization of the separation conditions, the average contents of the drugs in tablets or capsules were determined. After grounding the tablets in a mortar, a powder equivalents to 12.5 mg of each active substance (46.89 mg of Bezamidin, 115.87 mg of Lipanor, 123.36 mg of Fenoratio and 70.66 mg of Gemfibral) were transferred to 25 ml volumetric flasks containing 15 ml of methanol. After adding the 12.5 mg of internal standard, suspensions were mixed by reciprocating shaker for 15 min and diluted up to volume with methanol, then filtered. The samples of resulting solutions were analyzed in optimal separation conditions (pH 10, 240 V/cm, 25 °C). The ratio between peak area of analyte and peak area of internal standard was used for calculation. Due to stable migration time, there was no need to use corrected peak areas.

3. Results and discussion

3.1. Method development

In a CE process pH of the separation buffer, the voltage and temperature have very important roles. Thus, the effect of these conditions on the separation was studied (Fig. 1). It was observed, that below pH 6, the migration time increased rapidly due to lack of ionisation. Consequently, the drugs had not migrated from the capillary during the hour (maximum time of electropherogram measurement). In investigated pH range (6–11), the selectivity has not differed significantly, the best separation and peak shape was achieved at pH 10.

Next, we have examined effect of separation voltage at pH 10, in range 40–240 V/cm. The selectivity was not changing in whole range, only migration times were increasing when voltage was reduced. So, we have chosen maximum possible voltage (240 V/cm) for further analysis. The corresponding current was about 180 μA.

The temperature effect was not significant. In range 15–35°C, separation at pH 10 and 240 V/cm was quite similar and therefore the ambient 25 °C temperature was chosen.

Optimal electropherogram of fibrates obtained in above conditions, is presented in Fig. 2. The compounds without electrical charge (fenofibrate and clofibrate) are migrating together at void time, being successfully separated from the other compounds, which were also separated between themselves. The analysis time does not exceed 10 min.

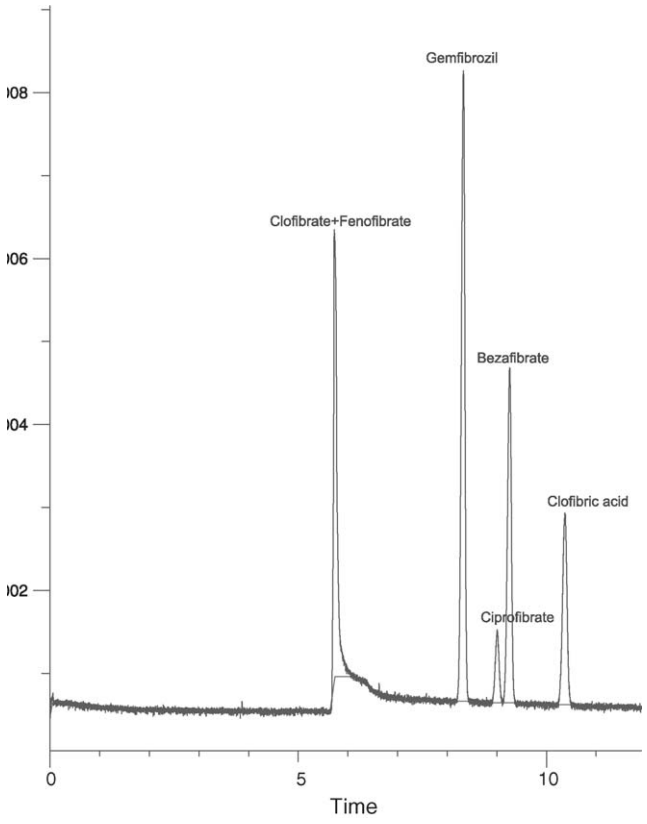

Fig. 2. Optimal electropherogram of fibrates (pH 10, 240 V/cm, 25 °C).

3.2. Quantification

Once the conditions for separation were established, they were applied to quantitate bezafibrate, ciprofibrate and gemfibrozil in pharmaceutical formulations. Clofibric acid was chosen as internal standard.

Calibration curves were constructed in range 0.2–0.8 mg/ml for all drugs. Solutions were prepared independently and measured five times for each concentration to perform complex statistical evaluation of proposed method. The whole statistics was calculated and graphed using R-project open source software [15] and depicted in Fig. 3.

The coefficient of determination, R^2 is most popular measure applicable to linear and polynomial regression. But this coefficient has low cognitive value in linear calibration, because high R^2 can be obtained by applying linear least squares method to significantly curvilinear data [14]. So we have decided to perform more complex evaluation to test linearity of calibration. All three curves proved to be linear by means of all common statistic tests used in this case, as shown in Table 1:

(1) Residuals of regression did not present a visible trend and were randomly scattered.

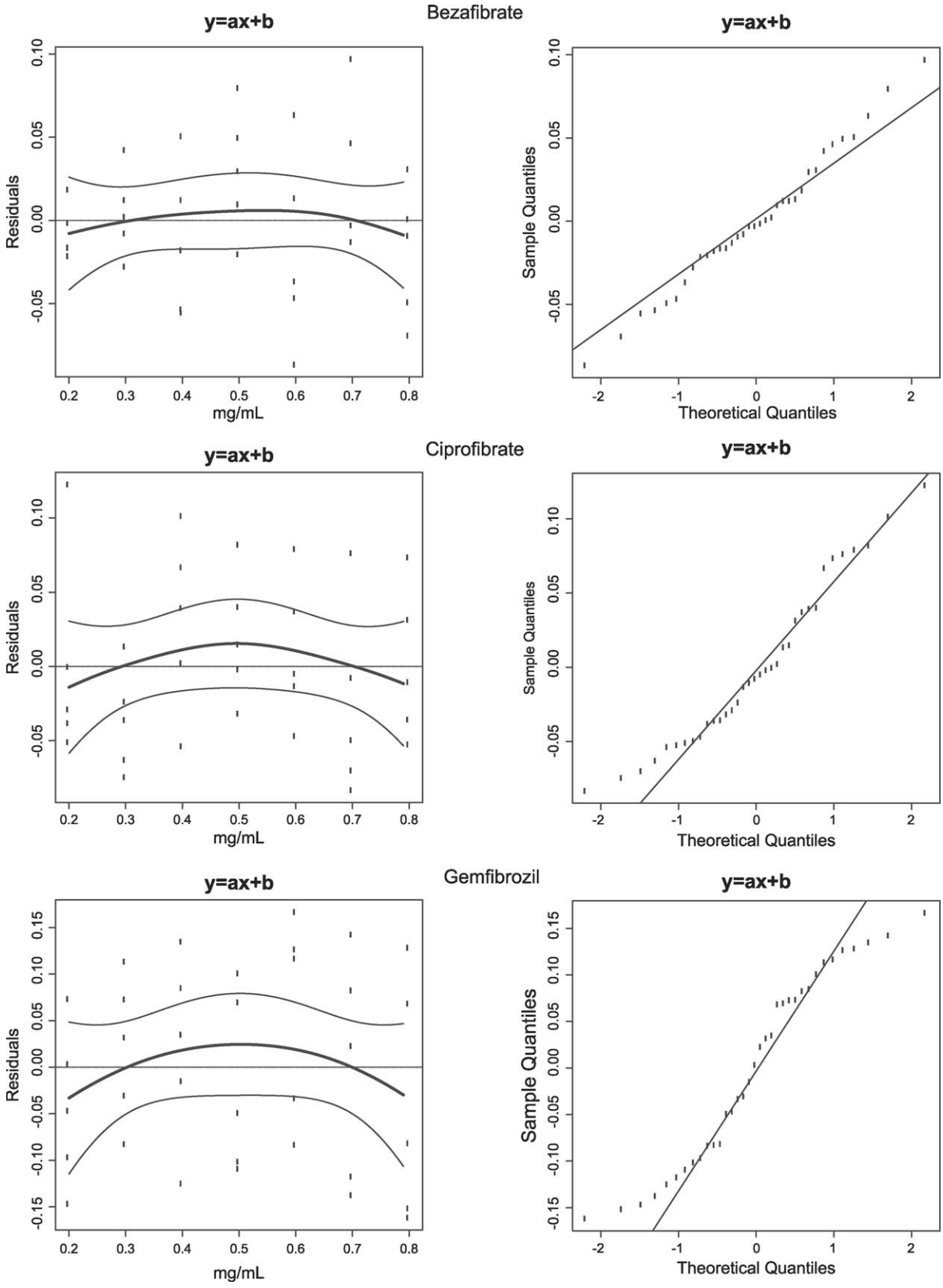

Fig. 3. Residual plots and quantile–quantile residual plots obtained during calibration procedure.

Table 1
Statistical evaluation of calibration: calibration equations, linearity and regression diagnostics

	Bezafibrate	Ciprofibrate	Gemfibrozil
Linear equation	$y = 2.162x + 0.038$	$y = 2.548x + 0.012$[a]	$y = 5.341x + 0.228$
Quadratic equation	$y = 2.350x + 0.0004$[a] $-0.187x^2$[a]	$y = 2.782x - 0.061$[a] $+0.234x^2$[a]	$y = 6.038x + 0.082$[a] $-0.696x^2$[a]
R^2 of linear model	0.9911	0.9899	0.9913
Significance of $p(cx^2)$	0.369	0.394	0.169
Lack-of-fit test	1.49 ($p = 0.22$)	1.97 ($p = 0.11$)	0.74 ($p = 0.60$)
Mandel's test	0.82 ($p = 0.36$)	0.74 ($p = 0.39$)	1.97 ($p = 0.16$)
Shapiro–Wilk test on residuals	0.988 ($p = 0.96$)	0.949 ($p = 0.11$)	0.944 ($p = 0.07$)

[a] Insignificant coefficient ($p > 0.05$).

Table 2
Statistical evaluation of results: content, recovery with its linearity, precision and fortified samples

		Bezafibrate	Ciprofibrate	Gemfibrozil
Content	Mean	204.85	97.32	456.81
	Recovery	102.42%	97.32%	101.51%
Precision	Repeatability	6.44%	4.01%	6.11%
	Reproducibility	9.51%	5.52%	11.15%
Accuracy	CI	161.06–248.64	82.56–112.09	393.15–520.47
	t-value	0.477($p = 0.68$)	−0.780($p = 0.51$)	0.460($p = 0.69$)
Recovery curve[a]	a	0.971	0.947	1.032
$y = ax + b$	*b*	0.010	0.019	−0.018
	R	0.9826	0.9977	0.9953
Fortified sample (50%)	Content	219.23	105.87	479.97
	Recovery	109.62%	105.87%	106.66%
	R.S.D.	16.14%	5.27%	13.25%
	t-Value	1.215($p = 0.29$)	2.352($p = 0.08$)	1.054($p = 0.35$)
Fortified sample (100%)	Content	185.37	104.19	442.86
	Recovery	92.68%	104.19%	98.41%
	R.S.D.	12.70%	5.31%	14.08%
	t-Value	−1.390($p = 0.23$)	1.694($p = 0.16$)	−0.256($p = 0.81$)
Fortified sample (150%)	Content	205.87	105.18	456.10
	Recovery	102.94%	105.18%	101.36%
	R.S.D.	7.03%	6.27%	10.48%
	t-Value	0.907($p = 0.41$)	1.754($p = 0.15$)	0.285($p = 0.78$)

[a] The intercept coefficient is insignificant at 95% confidence level.

(2) The Shapiro–Wilk test for normality did not reject the hypothesis that residuals are normally distributed.
(3) The ANOVA comparison of variance of regression residuals, called "Lack-of-fit" test, or "a priori" test did not reject hypothesis of linearity.
(4) The ANOVA between linear and quadratic model fitted to the same data, called Mandel's fitting test or "a posteriori" test was also below the critical value, and estimator related to quadratic term was insignificant.

3.3. Validation of the assay

After the calibration process the CE method was applied to determination of commercial formulations. Due to elution of fenofibrate and clofibrate at void time (they are uncharged), we decided to quantitate the other (charged) drugs. Fig. 4 shows chromatograms of samples. The quantitation results are given in Table 2.

The six samples, independently weighted and extracted, were quantified for statistical validation. In all cases the *t*-test for one mean (null hypothesis: population mean is equal to quantity declared by manufacturer) proved adequate accuracy. Precision was measured in two ways: by measuring the same sample six times (repeatability), and measuring peaks from six independent samples (reproducibility).

The test of recovery linearity and test of accuracy by analyzing fortified samples are also required to development of precise and repeatable CE assays. The linearity of recovery was checked by weighting different amounts of powdered tablet (or capsule) mass (which resulted in 0.3, 0.4, 0.5, 0.5 and 0.7 mg/ml solutions). The recovery function constructed from obtained data had in all cases small insignificant intercept, slope very close to 1 and linearity similar to 0.999.

Accuracy was tested by measuring fortified samples containing standard solutions and tablet extracts in different fractions (50, 100 and 150% of fortification). The results were homogenic

Table 3
Comparison of precision and accuracy of elaborated CE method with other previously elaborated methods for determination of fibrates

Alternative methods	*F*-test		*t*-Test		*U*-test	
	F	*p*	*t*	*p*	*W*	*p*
Bezafibrate						
Densitometry	0.97	0.97	−0.75	0.47	13	0.48
Videodensitometry	4.06	0.15	−1.44	0.19	10	0.24
HPLC	7×10^{-5}	[a]	−1.33	0.24	12	0.39
Derivative spectrometry	7.13	0.06	2.68	0.03	30	0.06
Ciprofibrate						
Densitometry	1.40	0.72	0.31	0.76	19	0.94
Videodensitometry	6.94	0.06	0.12	0.91	13	0.47
HPLC	8×10^{-5}	[a]	1.19	0.29	24	0.39
Derivative spectrometry	21.18	[a]	−0.28	0.79	17	0.94
Gemfibrozil						
Densitometry	2.42	0.35	−0.30	0.77	17	0.94
Videodensitometry	9.79	0.03	−2.83	0.03	5	0.04
HPLC	2×10^{-4}	[a]	−0.85	0.44	11	0.31
Derivative spectrometry	11.64	0.02	1.38	0.22	28	0.13

[a] *p*-Value is lower than 0.01.

and *t*-test showed no significant differences between them and declared amount.

3.4. Comparison with earlier work

We have also performed statistical comparison between CE method, and quantitative methods elaborated and published earlier: HPLC [16], densitometric and videodensitometric [17,18], and derivative spectrophotometry [19]. Precision between methods was compared using *F*-test. Accuracy was compared by *t*-test and *U* (Mann–Whitney) test, considering first or second test according to difference in precision. The results are presented in Table 3. We had no possibility to compare accuracy and precision of our method with methods given by Zarapkar et al. [7] and Li et al. [8]. The abstracts of their papers give no information about these parameters and the original article is written in Indian or Chinese.

The precision of elaborated method is not very high, because the analyzed compounds are practically insoluble in aqueous solvents, like neutral, acid or basic electrolytes. This forces the analyst to use methanol as extracting medium (it is the best solvent for them) and spiking methanolic extracts of tablets. The introductory experiments showed, that spiking standard amount of methanolic solutions results in very instable current, due to creation of a gap in circuit. Addition of salts to methanol improved this behavior, but resulted in significantly unstable migration times. The best solution was spiking smaller amounts of methanolic solutions without electrolyte, which resulted in stable migration times and best possible precision, slightly worse than expected, but not differing significantly from densitometry or videoscanning or derivative spectrometry (except derivative spectrometry of ciprofibrate).

The accuracy of CE method is comparable with all methods, and even better than derivative spectrometry of bezafibrate and videoscanning of gemfibrozil.

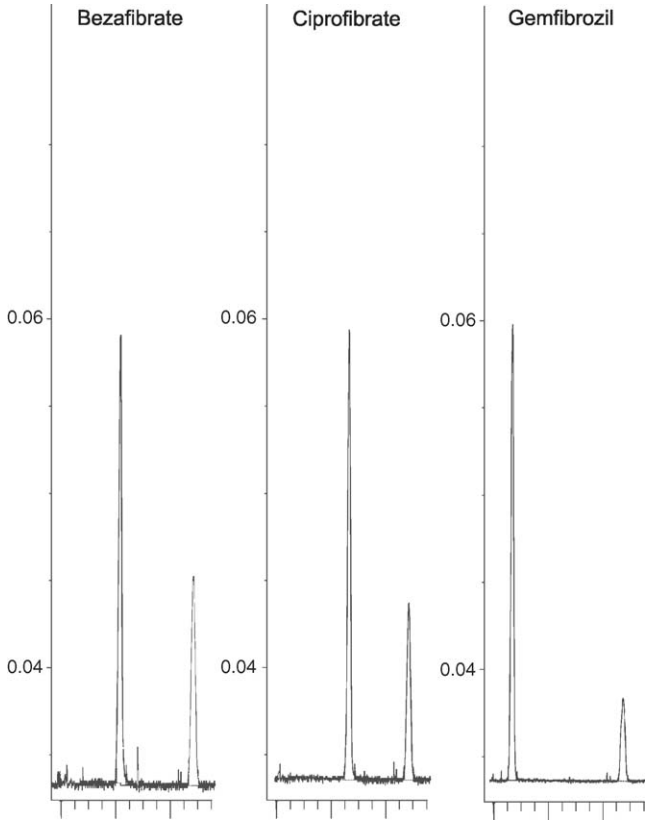

Fig. 4. Sample electropherograms obtained during analysis of pharmaceuticals.

4. Conclusion

Rapid, simple and accurate method for separation of fibrates and quantitation of them in tablets and capsules was elaborated and validated. This method allows to identify and separate charged fibrates, and quantitate bezafibrate, ciprofibrate and gemfibrozil with enough precision and good accuracy.

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