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Determination of nine β-blockers in serum by micellar electrokinetic capillary chromatography

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Abstract

β-Adrenergic blocking agents are used for the treatment of angina pectoris, cardiac arrythmia, hypertension, anxiety attacks, thyrotoxicosis, migraine and glaucoma. Owing to their sedative effect, they are also used as doping agents in sport. All β-blockers have an alkanol amine side chain terminating in a secondary amino group in their structure. The pK_a values vary from 9.2 to 9.8. Because some β-blockers are hydrophilic and some lipophilic, simultaneous determination is difficult. In this work, a method based on micellar electrokinetic capillary chromatography (MECC) was developed for the separation and determination of β-blockers in serum. The phosphate buffer 0.08 *M* (pH 6.7) solution contained 15 m*M* N-cetyl-N,N,N-trimethylammonium bromide. Nine parent β-blockers could be separated in a single run and the concentrations determined by internal standard (ephedrine) method. The simple clean-up procedure consisted of enzyme hydrolysis (*Helix pomatia*), protein precipitation, and filtration through 0.5-μm PTFE membranes. The MECC method exhibited good repeatability and a linear range of 75–300 μg/ml. The method was successfully applied after concentration to the determination of propranolol in real samples.

1. Introduction

Micellar electrokinetic capillary chromatography (MECC), which is a form of capillary zone electrophoresis (CZE) relying on an ionic micellar solution system, has lately become recognized as a technique suitable for the separation and determination of small neutral molecules and charged compounds [1]. In MECC, solutes are distributed between the micelles and aqueous phase and simultaneously separated electrophoretically according to their mobilities. MECC is the most widely used capillary electrophoresis method for drug analysis in biological matrices [2–8].

β-Adrenergic blocking agents are clinically used to treat angina pectoris, cardiac arrythmia, hypertension, anxiety attacks, thyrotoxicosis, migraine and glaucoma [9]. Owing to their sedative effect, they are also used as doping agents by athletes [10]. Current methods for determining and identifying β-blockers have been developed over a period of several decades. Optical [11], GC [12,13] and GC–MS [14], HPLC [15], TLC [16], immunological [17] and radioreceptor assay [18,19] methods are the conventional approaches to determining β-blockers in biological fluids. In most cases a single compound is determined, with another β-blocker used as internal standard [17], which is a realistic approach if it can be assumed that two β-blockers are not ingested at the same time. Recently, it has become of interest to determine several parent β-blockers simultaneously [1,20].

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Some β-blockers are hydrophilic and others lipophilic. In addition, their relatively high pK_a values (pK_a 9.2–9.8) complicate the sample pretreatment and analysis. At physiological pH (pH 7.4), β-blockers exist as single cations [15], which enables their separation and determination by methods exploiting the different mobilities of analytes in an electrical field. The structures and pK_a values [21] of the studied β-blockers are presented in Fig. 1.

The general procedure for the separation of β-blockers from blood samples was introduced for propranolol in 1965 [22]. The sample was made alkaline with sodium hydroxide solution and extracted with an organic solvent, heptane with 1% (v/v) ethanol. After an acidic back-extraction the amount of the β-blocker was measured by fluorescence spectrometry [21]. The sample pretreatment method for GC that Walle et al. [23] published in 1975 introduced some changes in the materials used but not in the procedure. The same procedure was also adopted in 1986 for use in an HPLC method [24]. This means that the sample pretreatment method has remained virtually unchanged for almost thirty years, while at the same time analytical techniques and equipment have developed dramatically.

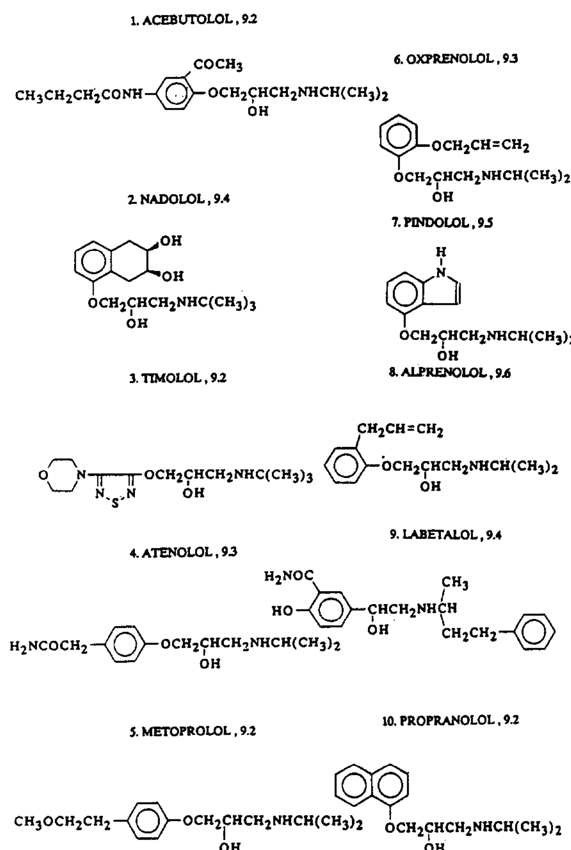

Fig. 1. Structures and pK_a values of β-blockers [14].

Urine is not so complex a matrix as plasma or serum as it does not normally contain significant amounts of proteins which tend to adsorb on to the capillary wall and interfere with the analysis. In our laboratory, β-blockers have been determined in human urine samples by MECC, with only dilution and filtration employed in the sample pretreatment [2].

Earlier we have studied the effects of instrumental parameters [25], pH [26] and organic modifiers [27] of the buffer solution on the separation and resolution of β-blockers in MECC. The results from these previous studies [25–27] are exploited in the optimization of the method we now describe for determining, in serum, nine parent β-blockers: acebutolol, alprenolol, atenolol, metoprolol, nadolol, oxprenolol, pindolol, propranolol and timolol. The spiked β-blockers were separated in a single run, after enzyme hydrolysis and protein precipitation. The method was validated for eventual application to real samples, obtained after oral administration of a β-blocker.

2. Experimental

2.1. Apparatus

MECC was performed in a 580 mm × 0.050 mm I.D. fused-silica capillary tube (Polymicro Technologies, White Associates, Pittsburgh, PA, USA) where 500 mm was the length from injector to detector. A Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) with laboratory-made temperature control unit was employed for the analyses. All experiments were done at 35°C. UV detection was at 214 nm.

Injections were carried out hydrostatically for 20 s and the running voltage was −27 kV at the injector end of the capillary. The data (peak height and migration times) were collected with an HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA).

2.2. Materials

The β-blockers were acebutolol hydrochloride, alprenolol hydrochloride, atenolol, labetalol hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxprenolol hydrochloride, pindolol, (*S*)-(−)-propranolol hydrochloride, timolol maleate, ephedrine hydrochloride (internal standard) and control serum type 1-A, all from Sigma (St. Louis, MO, USA). Acetonitrile, sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate, and N-cetyl-N,N,N-trimethylammonium bromide (CTAB) were from E. Merck (Darmstadt, Germany). The β-glucuronidase (EC 3.2.1.31) type H-1 was from *Helix pomatia* (416 800 I.U./g) (Separacor, France). Other reagents were of analytical grade and were used as received. A Water-I system from Gelman Sciences (Ann Arbor, MI, USA) was used for ion exchange of the distilled water. Samples and other solutions were filtered through Millex filters of 0.5 μm pore size from Millipore (Nihon Millipore, Kogyo K.K. Yonezawa, Japan). All the micellar buffer solutions were filtered through 0.45-μm membrane filters (Millipore, Molsheim, France) and degassed before use.

2.3. MECC buffer

The buffer was prepared from 0.08 *M* disodium hydrogenphosphate and 0.08 *M* sodium dihydrogenphosphate solutions containing 15 m*M* of CTAB. The pH of the buffer solution was adjusted to 6.7.

2.4. MECC procedure

To ensure a reproducible separation, before each injection the capillary was purged for 0.2 min with 5% (v/v) phosphoric acid, 0.5 min with water and 10 min with buffer solution.

2.5. Preparation of the serum samples

A 1-ml volume of serum was spiked with a solution containing an accurate amount of each β-blocker. The serum samples were hydrolyzed with β-glucuronidase enzyme at 80°C for 30 min. Proteins were precipitated by adding 700 μl acetonitrile to the samples, vortex-mixed for 15 min and centrifuged at 2004 *g* for 10 min. Ephedrine (165 μg/ml) was added as internal standard. The serum samples were passed through filters of 0.5 μm pore size and then analyzed.

Real human serum sample (2 ml) taken after 3 h after ingestion was prepared as described above except 900 μl of acetonitrile were added.

3. Results and discussion

Earlier studies have shown that MECC is a suitable technique for the determination of β-blockers in urine [2], and the use of organic buffer modifiers improves the separation [27]. Labetalol and propranolol were coeluted in the MECC method for serum described here, but with the addition of 1% (v/v) 2-propanol to the buffer solution they could be separated, too, so that altogether ten parent β-blockers were separated (Fig. 2). However, the resolution between atenolol and metoprolol was decreased by the addition of 2-propanol, and at higher concentrations labetalol and propranolol could not be quantified at the same time because they were again coeluted. The present study nevertheless clearly demonstrates that β-blockers can be determined in serum samples by MECC: Fig. 3 shows the baseline separation of nine β-blockers in 18 min. The interferences in the electropherogram are attributable to the serum background.

Like many other drugs, β-blockers tend to bind to the serum proteins and need to be released from them during the sample preparation. This can be done by enzyme hydrolysis, which is a better method than acidic or basic

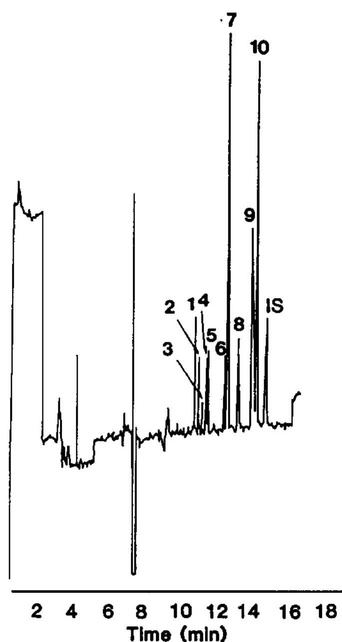

Fig. 2. Electropherogram of ten β-blockers at concentration 100 μg/ml (except for timolol 200 μg/ml) and ephedrine (internal standard, IS) at 165 μg/ml in 1% (v/v) 2-propanol modified buffer. Compound numbers can be found in Fig. 1 and separation conditions in the Experimental section.

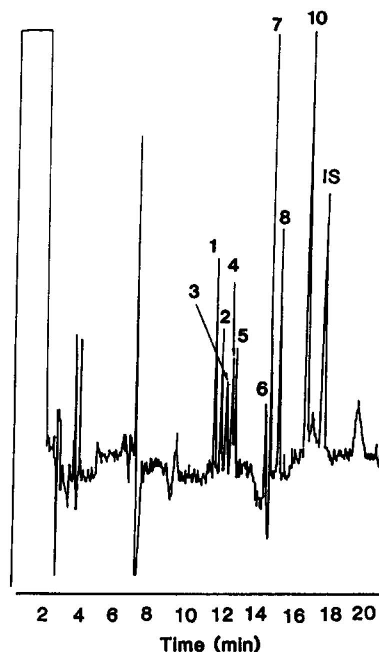

Fig. 3. Electropherogram of a serum sample containing nine β-blockers at concentration 150 μg/ml (except for timolol 300 μg/ml). For the identification of compounds see Fig. 1. Ephedrine (IS) is present at a concentration of 165 μg/ml. Separation conditions can be found in the Experimental section.

hydrolysis since the latter tend to decompose β-blockers. Sample clean-up is further important because the serum proteins tend to adsorb to the capillary wall and disturb the analysis. The simplest way to overcome this problem is to add organic solvent or sodium chloride to the sample. Acetonitrile was successfully used to precipitate the proteins from serum samples. Despite the requirement for two sample pretreatment steps, the method we describe is simpler and less time consuming than earlier methods [21,23,24]. Recovery for the β-blockers at 150 μg/ml (except for timolol 300 μg/ml) varied from 49% (nadolol) to 80% (alprenolol), as shown in a comparison of the peak height/migration time values of standard samples extracted from serum with the peak height/migration time values of unextracted standards.

Linearity was tested over the range 75–300 μg/ml. The correlation coefficients of the linearity curves varied from 0.986 to 0.997 (Table 1) and can be regarded as satisfactory.

Table 1
Linearity of the method in the range 75–300 μg/ml (timolol 150–600 μg/ml)

Compound	r	a	b
Acebutolol	0.986	0.22	0.01
Nadolol	0.991	0.17	0
Timolol	0.996	0.15	0
Atenolol	0.997	0.16	0
Metoprolol	0.997	0.05	0.01
Oxprenolol	–	–	–
Pindolol	0.997	1.03	0.04
Alprenolol	0.995	0.01	0.01
Propranolol	0.997	−0.20	0.03

Analyses were performed using 165 μg/ml of ephedrine as the internal standard. The concentrations of β-blockers used in the determination of the linear range were 75, 100, 125, 175, 250 and 300 μg/ml (for timolol 150, 200, 250, 350, 500 and 600 μg/ml). r = Correlation coefficient. The equation for the straight line is $y = bx + a$, where a is the intercept of the y axis and b the slope.

The linearity values for oxprenolol are missing because it sometimes coeluted with pindolol. Furthermore, the baseline penetration in the electropherogram caused by the serum matrix disturbed the measurement of oxprenolol (Fig. 3). The detection limit varied from 1 μg/ml for propranolol to 50 μg/ml for timolol (determined as $3 \times S/N$). The large difference between these values is explained by the considerable variation in the UV-absorption properties of β-blockers.

Repeatability of the method was determined at two levels: 150 and 250 μg/ml. Relative standard deviations (R.S.D.s) varied from 4.5 to 15.8% ($n = 6$) at the 150 μg/ml and from 4.2 to 12.3% ($n = 6$) at the 250 μg/ml level (Tables 2 and 3). The relatively high R.S.D.s are thought to be due to the injection technique and the complex serum matrix, but the values are still at acceptable level. The repeatability of the serum method is not as good as the corresponding method for urine [2], even though the urine samples were analyzed without temperature control.

The method can be applied to real samples. Propranolol was separated from a real human serum sample at μg/ml level (Fig. 4). Since the therapeutical blood level of propranolol is generally 1–500 ng/ml [28], the method is not sensitive enough as such for routine analysis. However, a concentration step can easily be included in the sample pretreatment procedure [*e.g.*, the acetonitrile portion used for protein precipitation can be evaporated to a smaller volume (even to 30 μl)], so that therapeutical serum samples

Table 2
Repeatability of the method at the level 150 μg/ml (timolol 300 μg/ml)

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	1.42	0.01	7.0
Nadolol	0.93	0.04	4.5
Timolol	0.60	0.07	11.8
Atenolol	0.74	0.09	11.8
Metoprolol	0.95	0.10	10.4
Oxprenolol	0.67	0.05	7.3
Pindolol	10.1	0.98	9.7
Alprenolol	1.32	0.21	15.8
Propranolol	4.45	0.57	12.9

Analyses were performed using 165 μg/ml of ephedrine as the internal standard. χ = Mean ($n = 6$) of (peak height/migration time of compound)/(peak height/migration time of internal standard); S.D. = standard deviation; R.S.D. = relative standard deviation.

Table 3
Repeatability of the method at the level 250 μg/ml (timolol 500 μg/ml)

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	1.74	0.12	6.7
Nadolol	1.11	0.08	6.8
Timolol	0.71	0.07	9.2
Atenolol	0.93	0.04	4.2
Metoprolol	1.29	0.05	3.9
Oxprenolol	0.59	0.07	12.3
Pindolol	11.20	0.69	6.1
Alprenolol	2.08	0.15	7.3
Propranolol	6.25	0.48	7.8

See Table 2.

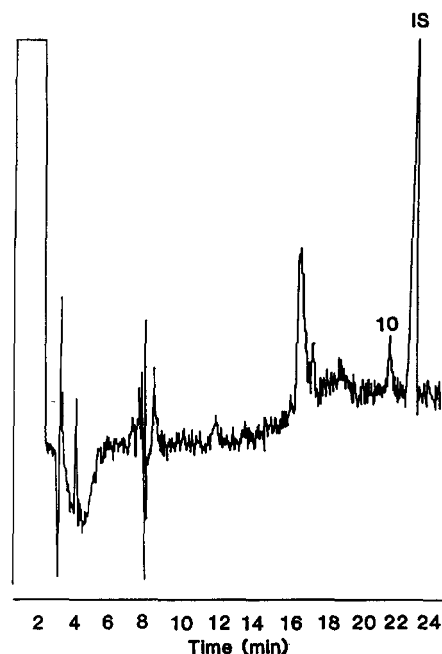

Fig. 4. Electropherogram of a real human serum sample containing propranolol (10). The sample was taken 3 h after ingestion. IS is ephedrine (165 μg/ml). Separation conditions can be found in the Experimental section.

containing β-blockers can be analyzed with the described MECC method.

4. Acknowledgement

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5. References

[1] S. Terabe, *Trends Anal. Chem.*, 8 (1989) 129.
[2] P. Lukkari, H. Sirén, M. Pantsar and M.-L. Riekkola, *J. Chromatogr.*, 632 (1993) 143.
[3] W. Thormann, P. Meier, C. Marcolli and F. Binder, *J. Chromatogr.*, 545 (1991) 445.
[4] R. Weinberg and I.S. Lurie, *Anal. Chem.*, 63 (1991) 823.
[5] P.G. Pietta, P.L. Mauri, A. Rava and G. Sabbatini, *J. Chromatogr.*, 549 (1991) 367.
[6] P. Lukkari, J. Jumppanen, K. Jinno, H. Elo and M.-L. Riekkola, *J. Pharm. Biomed. Anal.*, 10 (1992) 561.
[7] H. Nishi, T. Fukuyama and M. Matsuo, *J. Chromatogr.*, 515 (1990) 245.
[8] W. Thormann, A. Minger, S. Molteni, J. Gaslavska and P. Gebauer, *J. Chromatogr.*, 593 (1992) 275.
[9] R.G. Shanks, *Trends Pharm. Sci.*, 5 (1984) 451.
[10] J. Park, S. Park, D. Lho, H.P. Choo, B. Chung, C. Yoo, H. Min and M.J. Choi, *J. Anal. Toxicol.*, 14 (1990) 66.
[11] R. Swan, in I. Sunshine (Editor), *Methodology in Analytical Toxicology*, CRC Press, Cleveland, OH, 1975, p. 328.
[12] M. Ervik, *Acta Pharm. Suecica*, 6 (1969) 393.
[13] M. Ervik, K. Kylberg-Hanssen and L. Johansson, *J. Chromatogr.*, 381 (1986) 168.
[14] D.A. Garteiz and T. Walle, *J. Pharm. Sci.*, 61 (1972) 1728.
[15] K.V. Buhsing and A. Garbe, *J. Chromatogr.*, 382 (1986) 215.
[16] D.B. Jack, S. Dean and M.J. Kendall, *J. Chromatogr.*, 187 (1980) 277.
[17] K. Chegmidden, M.R. Pirio, P. Singh, J.B. Gushaw, J.G. Miller and R.S. Schneider, *Clin. Chem.*, 24 (1978) 1056.
[18] L.D. DeLeve, L. Endrenyi and F.H.H. Leenen, *J. Clin. Pharmacol.*, 25 (1985) 182.
[19] M. Ahnoff, M. Ervik, P.-O. Lagerstrom, B.-A. Persson and J. Vessman, *J. Chromatogr.*, 340 (1985) 73.
[20] H. Sirén, M. Saarinen, S. Hainari, P. Lukkari and M.-L. Riekkola, *J. Chromatogr.*, 632 (1993) 215.
[21] V. Marko, in V. Marko and R.A. de Zeeuw (Editors), *Determination of Beta-Blockers in Biological Material*, Elsevier, Amsterdam, 1989, p. 77.
[22] J.W. Black, W.A.M. Duncan and R.G. Shanks, *Br. J. Pharmacol.*, 25 (1965) 577.
[23] T. Walle, J. Morrison, K. Walle and E. Conradi, *J. Chromatogr.*, 114 (1975) 351.
[24] Y. Yamamura, K. Uchino, H. Kotaki, S. Isozaki and Y. Saitoh, *J. Chromatogr.*, 374 (1986) 311.
[25] P. Lukkari, A. Ennelin, H. Sirén and M.-L. Riekkola, *J. Liq. Chromatogr.*, 16 (1993) 2069.
[26] P. Lukkari, H. Vuorela and M.-L. Riekkola, *J. Chromatogr. A*, 652 (1993) 451.
[27] P. Lukkari, H. Vuorela and M.-L. Riekkola, *J. Chromatogr. A*, 655 (1993) 317.
[28] T. Trnovec and Z. Kállay, in V. Marko and R.A. de Zeeuw (Editors), *Determination of Beta-Blockers in Biological Material*, Elsevier, Amsterdam, 1989, p. 25.