Determination of ten P-blockers in urine by micellar electrokinetic capillary chromatography

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ABSTRACT

β-Adrenergic blocking agents are of therapeutic value in the treatment of migraine and various cardiovascular disorders (angina pectoris, cardiac arrythmia, hypertension). Owing to their sedative effect, they are also used as doping agents in sport. A characteristic feature of β-blockers is the alkanolamine side-chain terminating in a secondary amino group. The pK_a values vary from 9.2 to 9.8. Because some p-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. The 0.08 M phosphate buffer (pH 7.0) solution contained 10 mM N-cetyl-N,N,N-trimethylammonium bromide (CTAB). Ten parent P-blockers in human urine could be separated in a single run and determined quantitatively by the internal standard (2,6-dimethylphenol) method. Neither endogenous compounds in urine nor caffeine and its metabolites interfered with the analysis. The clean-up procedure for urine consisted of a simple filtration through 0.5-μm PTFE membranes. The MECC method exhibited good repeatability and a linear range of 25-150 μg/ml. The method was applied to determination of oxprenolol in real samples.

INTRODUCTION

The determination of drugs in biological fluids is growing in importance owing to the need to understand better the biochemical effects of drugs and the continuing development of more selective and effective drugs.

Methods for the screening and identification of P-blockers have been studied over several decades. TLC, HPLC and high-resolution GC-MS [1,2] are the conventional approaches after the drugs have been isolated from the matrices. Although these methods meet the demands of selectivity and sensitivity, the character of /?-blockers complicates the necessary preparation of biological fluids for their determination. Some /I-blockers are lipophilic and others hydrophilic. In addition, their relatively high pK_a values (9.2--9.8), caused by hydrogen bonding

between the free electron pair on the nitrogen atom of the terminal amino group and the hydrogen atom of the β-hydroxyl group, complicate their adsorption and extraction. At physiological pH (pH 7.4), β-blockers exist as single cations [3], which permits their separation and determination by methods based on ion-pair and micellar formations.

Capillary zone electrophoresis (CZE) has developed rapidly since Jorgenson and Lukacs [4,5] first realized the advantages of using narrow-bore (I.D. < 100 μm) silica capillaries. In CZE, charged particles can be separated on the basis of differences in their electrophoretic mobilities (μ_{ep}). Electroosmotic flow (EOF), which occurs in an electrolyte-filled capillary under an electric field, has a flat flow profile and mobility μ_{eo}; normally $\mu_{eo} > \mu_{ep}$. This effect forces all solutes (anionic, cationic and neutral) to migrate towards the cathode end of the capillary. Even anionic compounds are carried to the cathode as the EOF is much stronger than the μ_{ep} of charged particles. Under the influence of EOF, neutral particles migrate together at the velocity of the electrolyte.

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Micellar electrokinetic capillary chromatography (MECC), which is an adaptation of CZE, was described by Terabe *et al.* in 1984 [6]. In MECC, an ionic surfactant added to the electrolyte facilitates the separation of neutral particles, something that is not achieved by CZE. Although MECC is useful for the separation of electrically neutral compounds, it is also effective for separating ionic compounds, which, because of their similar electrophoretic mobilities, are not adequately resolved by CZE [7,8]. It is thought that chromatographic distribution principles are involved in MECC. Also, MECC has been used in drug analyses [9–12].

In this paper we present an MECC method for the determination of ten parent /?-blockers. The separation mechanism is based on the differential partitioning of analytes between an electroosmotically purged aqueous 0.08 M phosphate buffer phase and an electrophoretically retained cetyltrimethylammonium (10 mM) micellar pseudo-phase. With this method the ten parent P-blockers (spiked) in urine were separated during a single run, after sample preparation consisting only of filtration of the urine. The linear concentration ranges were studied for eventual application of the method to real samples obtained after oral administration of the drug. In addition, the possible interference of caffeine was investigated.

EXPERIMENTAL

Apparatus

MECC was performed in a 68 cm × 50 μm I.D. fused-silica capillary tube (White Associates, Pittsburgh, PA, USA) where 600 mm was the effective length for separation. A Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) was employed. Detection was at 214 nm. All experiments were done at ambient temperature. Injections were made hydrostatically for 30 s and the running voltage was -26 kV at the injector end. The data (peak height) were collected with an HP 3392A integrator and the identification of oxprenolol and its hydroxy-substituted metabolites was performed with CC-MS using a Hewlett-Packard (Avondale. PA, USA) Model 5890A gas chromatograph and a Hewlett-Packard Model 5989A single-stage quadrupole mass spectrometer.

Materials

The β-blockers studied were acebutolol hydrochloride, alprenolol hydrochloride, atenolol, labetalol hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxprenolol hydrochloride, pindolol, (S)-(–)-propranolol hydrochloride and timolol maleate, all from Sigma (St. Louis, MO, USA). 2,6-Dimethylphenol (internal standard), sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate and N-cetyl-N,N,N-trimethylammonium bromide (CTAB) were obtained from Merck (Darmstadt, Germany) and were used as received. Other reagents used in the development of the method were of analytical-reagent grade and were used as received. Distilled water was purified through a Water-I system from Gelman Sciences (Ann Arbor, MI, USA). All the micellar buffer solutions were filtered through 0.45-μm membrane filters (Millipore, Molsheim, France) and degassed before use. Samples and other solutions were filtered through Millex filters of 0.5-μm pore size (Nihon Millipore, Yonezawa. Japan).

Procedure

To ensure reproducible separations, before each injection the capillary was purged for 2 min with the buffer solution.

Preparation of spiked human urine samples

A human urine pool was diluted with water (1:2) and the mixture was spiked with a solution containing an accurately measured amount of each P-blocker. The urine samples were filtered through filters of 0.5-μm pore size and then analysed.

RESULTS

Optimization of MECC conditions

A standard solution of the ten B-blockers was prepared for optimization of the MECC conditions in the absence of the matrix. The elution order of the compounds was studied by individual spiking, but all ten p-blockers were always included in the solution. A pH of 7.0 produced by phosphate salts was chosen for the eluent on the basis of past experience in the analysis of &blockers by ion-pair liquid chromatography.

The parameters investigated were the concentrations of phosphate and the micelle former. CTAB.

which normally is used as the ion-pair former in HPLC, and the applied voltage. Phosphate concentrations from 0.05 to 0.15 M and CTAB concentrations of 5, 8, 10, 12 and 15 mM were studied. On the basis of their simultaneous effect on the resolution, we chose concentrations of 0.08 M phosphate buffer and 10 mM CTAB. Under these buffer and micellar conditions the best value for the voltage was − 26 kV. The analysis could thus be performed and almost a baseline separation achieved with the lowest current of the apparatus.

Choice of internal standard

An internal standard was required for quantitative MECC analysis because the ambient temperature and the temperature in the sample space influence the migration times. The negative temperature effect could have been reduced if our CZE equipment had included a temperature control unit.

A suitable internal standard was not easily found as two main requirements had to be met: it had to resemble the structure of the j&blockers, *i.e.,* it had to have at least either a hydroxyl or an amino group, and it had to migrate in the middle of the drug zone. 2,6-Dimethylphenol, met both of these criteria and was chosen as the internal standard (ISTD). Its resolution from drugs migrating nearby was complete. A further advantage of 2,6-dimethylphenol is that it is unlikely to be found in the human body. Diphenylamine and probenecid were also evaluated as internal standards but were found to be unsuitable, the former because it did not absorb well enough at 214 nm and the latter because it migrated together with timolol and atenolol.

Samples and technique

The samples were prepared by adding various amounts of the standard mixture to human urine diluted with water (1:2). The internal standard was added. The only preparation applied to the samples was filtration. A blank pooled urine sample obtained from people drinking coffee or tea, but not smoking and not using drugs, was prepared simultaneously, without internal standard addition.

As can be seen in Fig. 1, neither endogenous compounds in urine nor caffeine and its metabolites interfered with the analysis as they migrated to the detector within 5 min. After their migration was complete. all ten P-blockers and the internal stan-
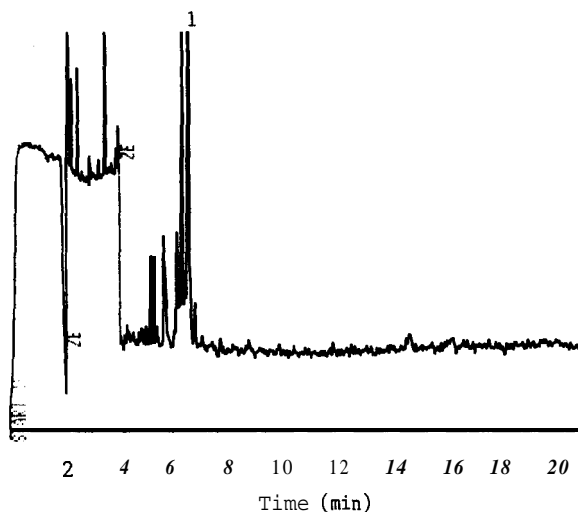

Fig. 1. Electropherogram of blank human urine sample in 0.08 M sodium phosphate buffer containing 10 mM CTAB. Capillary, 68 cm × 50 μm I.D.; pH, 7.0; hydrodynamic injection mode, 30 s at 10 cm height; detection, UV at 214 nm; applied voltage, -26 kV; temperature, ambient. In the electropherogram caffeine migrates in zone 1.
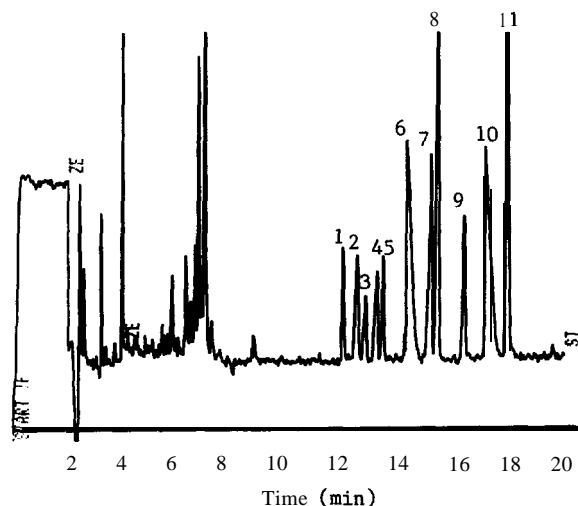

Fig. 2. Electropherogram of a human urine sample in 0.08 M sodium phosphate buffer with 10 mM CTAB. Capillary, 68 cm ×50 μm I.D.; pH, 7.0; hydrodynamic injection mode, 30 s at 10 cm height; detection, UV at 214 nm; applied voltage, − 26 kV; temperature, ambient. The concentrations of the β-blockers were 75 μg/ml, except for timolol and 2,6-dimethylphenol (150 μg/ml). Elution order and relative retention times of the solutes: 1 = acebutolol (0.84); 2 = nadolol (0.87); 3 = timolol (0.90); 4 = atenolol (0.92); 5 = metoprolol (0.94); 6 = 2,6-dimethylphenol (1.00) (internal standard); 7 = oxprenolol (1.06); 8 = pindolol (1.08); 9 = alprenolol (1.15); 10 = labetalol (1.20); 11 = propranolol (1.26).
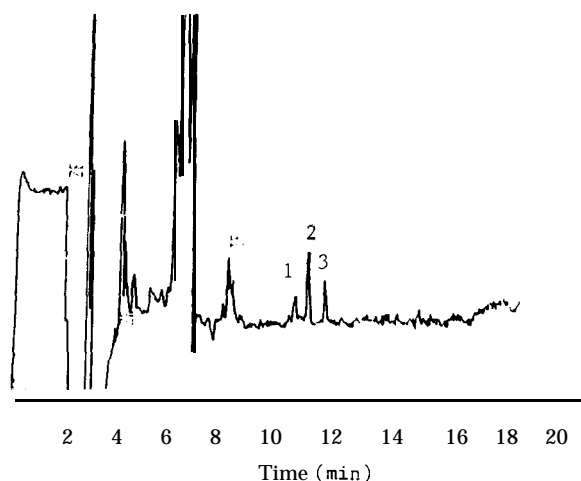
Fig. 3. Electropherogram of a real human urine sample after eightfold concentration by solid-phase extraction in 0.08 *M* sodium phosphate buffer with 10 m*M* CTAB. Capillary, 58 cm × 50 pm I.D.; pH, 7.0; hydrodynamic injection mode. 60 s at 10 cm height; detection, UV at 214 nm; applied voltage, − 26 kV; temperature, ambient. Peaks: 1 and 2 = hydroxy-substituted metabolites of oxprenolol; 3 = oxprenolol. The urine was taken 0–4 h after administration.

dard were eluted with high efficiency and resolution (Fig. 2) within 20 min. As can be seen in Fig. 1, the zone in which the P-blockers migrated was entirely free from other drugs and compounds.

The described method was also applied to the screening P-blockers in real samples. Oxprenolol and its two hydroxy-substituted metabolites were separated after eightfold concentration in less than 12 min from a human urine sample (Fig. 3). The compounds were identified using GC-MS.

The elution order of the drugs was determined by adding them one by one to a standard solution and was found to be acebutolol, nadolol, timolol, atenolol, metoprolol, 2,6-dimethylphenol, oxprenolol, pindolol, alprenolol, labetalol and propranolol. The detector responses of the drugs differed widely when their UV absorption was measured at 214 nm (one of the constant wavelengths in the apparatus). They absorb more strongly in the wavelength range 220-230 nm and the availability of this range would improve the determination.

Repeatability

Repeatability of the injections, which took 30 s at

TABLE I

REPEATABILITY OF INJECTION AT THE LEVEL OF 150 μg/ml (TIMOLOL 300 μg/ml)

Analyses were performed using *150* μg/ml of 2,6-dimethylphenol as the internal standard. χ is the mean ($n = 6$) of (peak height of compound)/(peak height of internal standard), S.D. the standard deviation and R.S.D. the relative standard deviation.

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	1.23	0.03	2.4
Nadolol	0.93	0.02	2.6
Timolol	0.24	0.02	8.2
Atenolol	0.68	0.01	2.0
Metoprolol	0.72	0.03	4.2
Oxprenolol	0.64	0.02	2.5
Pindolol	3.65	0.07	2.0
Alprenolol	0.99	0.03	2.6
Labetalol	1.35	0.04	2.1
Propranolol	4.18	0.09	2.3

a height of 10 cm, was studied at concentrations of 150 μg/ml, except for timolol, which was studied at 300 μg/ml. 2,6-Dimethylphenol (150 μg/ml) was added to ensure the repeatability of the injections. The relative standard deviations varied from 2.0 to 8.2% ($n = 6$) (Table I). These relatively high values are probably due to the hydrodynamic injection technique and the strong matrix effect.

The repeatability of the method was studied at two concentration levels, 2.5 and 150 μg/ml (50 and 300 μg/ml for timolol). The relative standard devia-

TABLE II

REPEATABILITY OF THE METHOD AT THE LEVEL OF 25 μg/ml (TIMOLOL 50 μg/ml)

Details as in Table 1. except $n = 5$.

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	0.34	0.01	2.8
Nadolol	0.35	0.02	5.6
Timolol	0.12	0.01	7.0
Atenolol	0.23	0.01	2.0
Metoprolol	0.25	0.01	5.1
Oxprenolol	0.23	0.01	3.8
Pindolol	0.67	0.04	6.3
Alprenolol	0.31	0.02	5.0
Labetalol	0.57	0.01	2.4
Propranolol	1.18	0.08	6.7

TABLE III

REPEATABILITY OF THE METHOD AT THE LEVEL OF 150 μg/ml (TIMOLOL 300 μg/ml)

Details as in Table I.

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	**0.88**	0.02	2.7
Nadolol	0.82	0.03	4.1
Timolol	0.31	0.01	2.7
Atenolol	0.60	0.02	3.2
Metoprolol	0.64	0.02	3.8
Oxprenolol	0.61	0.02	3.8
Pindolol	2.01	0.07	3.4
Alprenolol	0.86	0.03	3.8
Labetalol	1.26	0.04	3.3
Propranolol	3.67	0.11	2.9

tions varied from 2.0 to 7.0% (n = 5) at the 25 μg/ml (timolol 50 μg/ml) level and from 2.7 to 4.1% (n = 6) at the 150 μg/ml (timolol 300 μg/m) level (Tables II and III). The repeatability can be considered to be satisfactory.

Linearity

The linearity range for the compounds in human urine was 25-150 μg/ml, except for timolol, for

TABLE IV

LINEARITY OF THE METHOD IN THE RANGE 25-150 μg/ml (TIMOLOL 50–300 μg/ml)

Analyses were performed using 150 μg/ml of 2,6-dimethylphenol as the internal standard. The concentrations of /?-blockers in the determination of the linear range were 25, 50, 75 100 and 150 μg/ml (for timolol 50, 100, 150, 200 and 300 μg/ml). r is the correlation coefficient. The equation for the straight line is $y = bx + a$, where a is the intercept on the y-axis and b is the slope. The /I-blockers were eluted in the order listed.

Compound	r	a	b
Acebutolol	**0.998**	2.00	0.01
Nadolol	0.994	0.14	0.01
Timolol	0.997	0.05	0.02
Atenolol	0.994	0.09	0.01
Metoprolol	0.992	0.06	0.01
Oxprenolol	0.996	0.05	0.01
Pindolol	0.999	0.07	0.02
Alprenolol	0.998	0.06	0.01
Labetalol	0.998	0.33	0.01
Propranolol	1.000	0.12	0.03

which it was 50–300 μg/ml. At concentrations higher than 150 μg/ml, (for timolol higher than 300 μg/ml) the calibration graphs were parabolic. The correlation coefficients of the straight lines ranged from 0.992 to 1.000 (Table IV).

Detection limits

The detection limits for the drugs were calculated from electropherograms obtained using human urine spiked with the ten P-blockers. The detection limits were 10 μg/ml for all compounds except tim-0101, for which it was 20 μg/ml, with a signal-to-noise ratio of 3. This meant that, for detection at 214 nm, the concentration of the drugs in samples had to be at the μg/ml level before injection.

CONCLUSIONS

The described MECC method was successful for the simultaneous determination of acebutolol, nadolol, timolol, atenolol, metoprolol, oxprenolol, pindolol, alprenolol, labetalol and propranolol added to human urine. Under the conditions applied, the ten /I-blockers can be separated with high efficiency and resolution in less than 20 min. The repeatability is good when the sample contains an internal standard such as 2,6-dimethylphenol. Also, this MECC method is suitable for the determination β-blockers in real urine samples. The sample preparation method for isolation of the drugs is extremely simple and, unlike normal clean-up methods such as liquid-liquid and solid-phase extraction, it is not time consuming. Complicated clean-up techniques limit the number of routine analyses that a laboratory can handle, and transfer of extracts, evaporation and other procedures are likely to result in sample losses through adsorption.

The MECC method is highly practical as the samples do not require more than a simple filtration before injection. The use of GC- and LC–electron-capture detection and GC-MS techniques requires derivatization of analytes, which makes screening very expensive. Chromatographic separation is nevertheless commonly used for pindolol, propranolol, alprenolol, oxprenolol, practolol and timolol [13]. In some of the methods another P-blocker is used as an internal standard on the basis that patients are unlikely to be using two of the drugs together. In published methods, only a few drugs are deter-

mined simultaneously [14], which makes those methods unsuitable for screening.

The detection limits achieved were 10 μg/ml for all the β-blockers except timolol (20 μg/ml). In comparison, the detection limit for atenolol is as low as 2 ng/ml using HPLC with fluorescence detection [15]. However, the detection limits are at the μg/ml level for atenolol, metoprolol and labetalol in HPLC analysis with UV detection. Injection volumes can be very low with MECC, which should make it possible to preconcentrate the sample without taking larger volumes of the sample to the clean-up stage.

The MECC method offers an alternative to current methods for the screening of β-adrenoceptor antagonists. The poor sensitivity, the main disadvantage of the method as it now exists, might be overcome by preconcentration of the drugs. The effect of preconcentration, and of other pretreatment methods such as hydrolysis, will be studied and developed further.

REFERENCES

1 P. Lillsunde and T. Korte, *J. Anal. Toxicol.*, 15 (1991) 71.
2 M. Ahnoff, M. Ervik, P.-O. Lagerström, B.-A. Persson and J. Vessman, *J. Chromatogr.*, 340 (1985) 73.
3 M. Laxer, A. C. Capomacchia and G. E. Hardee, *Talanta*, 28 (1981) 973.
4 J. W. Jorgenson and K. D. Lukacs. *Anal. Chem.*, 53 (1981) 1298.
5 J. W. Jorgenson and K. D. Lukacs, *J. Chromatogr.*, 218 (1981) 209.
6 S. Terabe, K. Otsuka, K. Ichiakva. A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
7 A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger. *Anal. Chem.*, 59 (1987) 1021.
8 H. Nishi, N. Tsumagari, K. Kakimoto and S. Terabe, *J. Chromatogr.*, 465 (1989) 33 1.
9 W. Thormann. P. Meier, C. Marcolli and F. Binder, *J. Chromatogr.*, 545 (1991) 445.
10 W. Thormann, A. Minger, S. Molteni, J. Caslavska and P. Gebauer, *J. Chromarogr.*, 593 (1992) 275.
II P. Wernly and W. Thormann, *Anal. Chem.*, 63 (1991) 2878.
12 P. Lukkari, J. Jumppanen, K. Jinno, H. Elo and M.-L. Riekkola, *J. Pharm. Biomed. Anal.*, 10 (1992) 56 1.
13 Y.-G. Yee. P. Rubin and T. F. Blaschke, *J. Chromatogr.*, 171 (1979) 357.
14 P. M. Harrison, A. M. Tonkin and A. J. McLean, *J. Chromatogr.*, 339 (1985) 429.
15 D.-S. Lho, J.-K. Hong, H.-K. Peak, J. A. Lee and J. Park, *J. Anal. Toxicol.*, 14 (1990) 429.