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Ion-pair chromatography and micellar electrokinetic capillary chromatography in analyzing beta-adrenergic blocking agents from human biological fluids

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Abstract

Ion-pair chromatography (IPC) and micellar electrokinetic capillary chromatography (MECC) were used for the separation and determination of parent β -blockers from human biological fluids. In both these techniques, N-cetyl-N,N,N-trimethylammonium bromide (CTAB) was used as a buffer additive. In IPC, CTAB was an ion-pair former, and in MECC it was a micelle-forming surfactant. The effectiveness of the IPC method using methanol-gradient elution and that of MECC were compared for drug-spiked serum and urine samples. Detection was performed with a diode-array detector in the IPC method and with a 214-nm filter in the MECC technique. In both methods a phosphate buffer (pH 7.0) was used. In MECC the buffer solution contained 10 mM CTAB, while in IPC the CTAB concentration was decreased from 7 to 4 mM during the separation when a methanol gradient was used. The study showed that the IPC technique performed better for bioanalyses than the high-performance MECC technique, since in MECC UV detection presented a problem because of the low sample concentration. However, in MECC sample preparation was less time-consuming, using hydrolyzation and protein precipitation and, unlike the IPC technique, it did not require any liquid–liquid extraction step.

1. Introduction

Reversed-phase high-performance liquid chromatographic (RP-HPLC) separations and identifications of β -blockers after isolation from biological matrices have been developed in many laboratories as methods alternative to the derivatization of the drugs and their analysis by gas chromatography (GC). Time-consuming pretreatment steps of the biological fluids are needed for GC separations. However, the main advantage of the latter over other separation techniques is the simultaneous analysis of several

β -blockers, which may be difficult with RP-HPLC [1]. Drawbacks in RP-HPLC include the resolution efficiency and intensity problems at UV wavelengths, which have been surmounted in ion-pair chromatographic (IPC) separations [2].

Recently, many techniques for faster and easier sample pretreatment and analysis of β -blockers with HPLC have been developed [3,4]. On-line and off-line GC techniques [5–7] have also proved to be very practical, especially when automation of the procedure is not required or when only a few samples have to be analyzed. Among the latest analysis techniques for the components discussed here are the capillary

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electrophoretic (CE) methods developed by Lukkari et al. [8–11]. These methods can easily be automated. Another advantage is that, especially in urine matrix, no further clean-up steps other than filtration are needed. However, the CE techniques are not transferable to clinical bioanalyses without extra concentration steps due to the small sample amount in the capillaries, which reduces the response in UV detection.

Some HPLC studies have reported the simultaneous separation of more than two parent β -blockers [12]. The authors have mainly demonstrated the selectivity of the method used. In bioanalysis, a considerable amount of work has been done using RP-HPLC to screen one β -blocker. A different β -blocker was used as an internal standard [13]: it was assumed that the two β -blockers are unlikely to appear simultaneously in the same sample. However, in MECC all β -blockers available on the Finnish market can easily be analyzed within one electrophoretic run [8,9]. In MECC the separation of the analytes is based on the equilibrium partitioning between the micelles and the surrounding solution. This is possible when the electrolyte solution contains surfactant above the critical micelle-formation concentration (CMC), which is dependent on the concentrations of all the additives in the electrolyte used. Therefore, e.g. MECC with a cationic surfactant (CTAB) is highly effective for the analysis of drugs and their metabolites in body fluids [8–11,14,15]. Furthermore, when the analytes are ionized, their electrophoretic mobilities also affect the separations. In MECC the micelles form their own zone, which is the main difference between MECC and IPC. In IPC the ion-pair former (reversed-phase) is partly adsorbed onto a non-polar stationary phase in the chromatographic column, is partly free in the eluent and partly forms uncharged ion-pairs with the analytes by Coulombic forces.

With the IPC method described in this paper, we studied the simultaneous separation of six parent β -blockers (acebutolol, alprenolol, atenolol, metoprolol, oxprenolol and propranolol) and their mixture spiked in serum and in

urine. The method was based on reversed-phase ion-pair (RP-IP) formation. The ion-pair former was CTAB (N-cetyl-N,N,N-trimethylammonium bromide), the concentration of which varied during the gradient elution. With the MECC method described in this paper, we studied the simultaneous separation of ten parent β -blockers (acebutolol, alprenolol, atenolol, labetalol, metoprolol, nadolol, oxprenolol, pindolol, propranolol and timolol), spiked in serum and in urine, with an inside uncoated silica capillary using phosphate buffer (pH 7.0) containing CTAB as the surfactant. In both IPC and MECC, the only pretreatment for urine samples was filtration. The serum samples were hydrolyzed and proteins precipitated, after which the supernatant was solid-phase extracted (SPE). The studies demonstrate the suitability of IPC and MECC for the separation of β -blockers from biological matrices: IPC with diode-array detection (DAD) can be used for pretreated samples, but MECC with UV detection can reliably be used only when the analytes are concentrated before introducing them into the system.

2. Experimental

Capacity factors (k') were not calculated because in MECC the values are biased by uncertainties in the true ionic electrophoretic mobility [16]. In addition, it was difficult to find a reliable and detectable marker for the micelle mobility, i.e. one which is totally partitioned into the micelle under the MECC conditions used. In both IPC and MECC the resolution values (Eq. 1) and the effective theoretical plates per meter (Eq. 2),

$$R = \frac{t_{r,2} - t_{r,1}}{w_{1/2,1} + w_{1/2,2}} \quad (1)$$

$$N = 5.545 \left(\frac{t'_r}{w_{1/2}} \right)^2 \quad (2)$$

were calculated by the general half-width ($w_{1/2}$) method to compare these two methods more

accurately. The t_0 -values were determined by methanol in IPC and MECC.

2.1. Instrumentation

The liquid chromatograph was a Hewlett-Packard Model 1090 instrument equipped with an HP 1040A diode-array detector and an HP85B single-channel personal computer. An HP 9121 disc drive unit, a DPU multichannel integrator, an HP 3392A integrator and an HP 7470A plotter were used for data storage and reporting (Hewlett-Packard, Avondale, PA, USA). The column system used was an HP C₁₈ guard column (10 × 4.6 mm I.D., 5 μm) connected to a C8P-50 separation column (Asahipak, Japan, 150 × 4.6 mm I.D., 5 μm). Detection was performed at wavelengths 220, 230, 260 and 280 nm. The sample loop volume was 20 μl. The experiments were carried out at ambient temperature (23–25°C). Methanol-gradient elution was used in modifying the phosphate buffer (pH 7.0) containing 10 mM CTAB.

MECC was performed in 680 × 0.050 mm I.D. and 560 × 0.050 mm I.D. fused-silica capillary tubes (Polymicro Technologies, White Associates, Pittsburgh, PA, USA) with 600 mm and 480 mm, respectively, as the effective lengths for separation. A Waters Quanta 4000 capillary electrophoresis system (Millipore Corporation, Waters Chromatography Division, Milford, MA, USA) was employed. A wavelength of 214 nm was used for detection. All experiments were carried out at ambient temperature (ca. 25°C). Samples were injected hydrostatically for 30 s, and the running voltage was –26 kV. The current during the separations was 97 μA. The data (peak height) were collected with an HP 3392A integrator.

The pHs of the buffers used were adjusted with a Jenway 3030 pH meter connected to a Jenway electrode (Jenway, Felsted, UK) containing 4 M KCl and saturated calomel. Calibration of the electrode system was made with potassium hydrogen phthalate (0.05 M, pH 4.00) and sodium tetraborate (0.01 M, pH 9.81) solutions.

The urine samples were filtered through Sar-

torius Minisart NML sterile filter units (0.45 μm; Sartorius, Göttingen, Germany) or Millex filters (0.5 μm; Nihon Millipore, Kogyo K.K. Yonezawa, Japan).

All eluents in IPC and electrolyte solutions in MECC were filtered through Millipore filters (0.45 μm; Millipore, Molsheim, France) and degassed before use by ultrasonication.

2.2. Materials

The β-blocking agents used in these experiments were acebutolol hydrochloride, alprenolol hydrochloride, atenolol, labetalol hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxprenolol hydrochloride, pindolol, S-(–)-propranolol hydrochloride and timolol maleate, all from Sigma (Sigma Chemical, St. Louis, MO, USA). 2,6-Dimethylphenol (internal standard) in MECC, biphenylamine (internal standard) in IPC, sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate and N-cetyl-N,N,N-trimethylammonium bromide (CTAB) were from Merck (E. Merck, Darmstadt, Germany). Other reagents used in the method development were of analytical grade. All the reagents were used without further purification. Distilled water was ion-exchanged through a Water-I system from Gelman Sciences (Ann Arbor, MI, USA).

2.3. Preparation of standards

Standard stock solutions were prepared separately for each drug (1 mg/ml for IPC and 4 mg/ml for MECC) in methanol and in a mixture of methanol and water. Calibration reference solutions for biological fluids were prepared by spiking the urine blank or serum blank with each standard stock solution.

2.4. Sample preparation

For IPC the samples were prepared by adding 500 μl of each stock solution to 900 μl of serum (taken from human volunteers into Venoject tubes, Leuven, Belgium, VT-050 PZX, silicone initial coating, sizes 5 and 10 ml) or 900 μl of

drug-free urine (pooled). The urine samples were filtered before injection, but the serum samples were hydrolyzed with β -glucuronidase (EC 3.2.1.31) type H-1 from *Helix Pomatia* (416 800 units/g) enzyme at 60°C for 1 h before further clean-up steps [2,9]. After that, proteins were precipitated by adding methanol, centrifuged at 2000 g and the supernatant was cleaned up [2] with Supelclean LC-18 SPE tubes (3 ml; Supelco, Bellefonte, USA) before filtration and introduction onto the HPLC system.

For MECC a human urine pool taken from volunteers who were coffee or tea drinkers, but not drug users, was diluted with water (1:2, v:v). The mixture was spiked with a stock solution containing a known amount of each β -blocker. The urine samples were filtered using 0.5- μ m pore-size membranes and then analyzed by MECC. The serum samples were hydrolyzed with type H-1 enzyme as in the IPC samples in a sonification bath for 30 min. After this the samples were cleaned up with Supelclean LC-18 SPE tubes (3 ml) (Supelco).

2.5. Preparation of buffers

The buffers (pH 7.0) were made up of 0.1 M and 0.08 M sodium dihydrogen phosphate and 0.1 M and 0.08 M disodium hydrogen phosphate solutions. After mixing the two phosphate solutions to give pH of 7.0, 10 mM CTAB was added.

2.6. Instrumental procedures

In IPC, the preconditioning and the conditioning before each run were done by 0.1 M phosphate buffer containing 7.0 mM CTAB for 10 min. The drugs were eluted with methanol gradient in phosphate buffer. The methanol content was maintained at 30% for 4 min and was then increased to 45% at a rate of 5%/2 min, to 50% at 5%/2 min and finally to 60% at 10%/2 min. The CTAB concentration was decreased as follows: 7.0, 5.5, 5.0 and 4.0 mM during the four steps, respectively.

In MECC the conditioning procedure was much simpler than in IPC. The capillary was

only purged for 2 min with the phosphate buffer solution before each injection.

3. Results and discussion

In the IPC system an analytical method was developed to separate six β -blockers and the internal standard (biphenylamine, ISTD), all of which were spiked into biological fluids (serum and urine) in a single run after sample pretreatment before injection. The separated β -blockers in order of elution were: 1. atenolol, 2. acebutolol, 3. metoprolol, 4. oxprenolol, 5. alprenolol and 6. propranolol (Figs. 1 and 2). By using MECC, ten β -blockers were separated from urine, and their elution order was as follows: 1. acebutolol, 2. nadolol, 3. timolol, 4. atenolol, 5. metoprolol, 6. 2,6-dinitrophenol (ISTD), 7. oxprenolol, 8. pindolol, 9. alprenolol, 10. labetalol and 11. propranolol (Figs. 3 and 4). However, the MECC analyses of serum samples were performed without ISTD, because neither of the compounds (2,6-dinitrophenol, biphenylamine) was suitable for MECC. Because

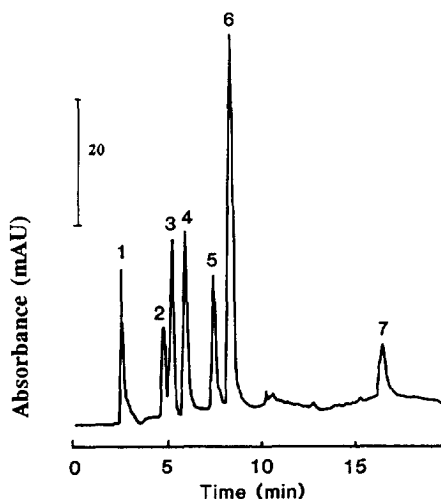


Fig. 1. Ion-pair chromatogram of human serum spiked with 670 ng of 1 = atenolol, 2 = acebutolol, 3 = metoprolol, 4 = oxprenolol, 5 = alprenolol, 6 = propranolol. Peak number 7 is the ion-pair former, CTAB. Separation conditions as described in the Experimental section. Detection at wavelength 280 nm.

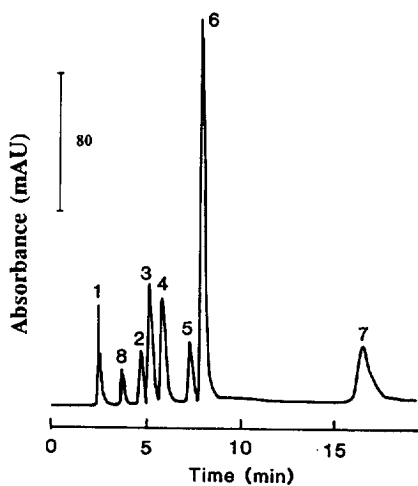


Fig. 2. Ion-pair chromatogram of human urine spiked with 250 ng of 1 = atenolol, 2 = acebutolol, 3 = metoprolol, 4 = oxprenolol, 5 = alprenolol, 6 = propranolol. Peak numbers 7 and 8 are the ion-pair former CTAB and caffeine, respectively. Separation conditions as described in the Experimental section. Detection at 260 nm.

the 2,6-dinitrophenol peak was split in serum samples, it was not suitable as an ISTD. Biphenylamine did not absorb intensively enough at 214 nm.

From Table 1 it can be seen that the resolution of the separated β -blockers in the IPC system is more effective when the drugs are isolated from serum than from urine. The reason for this is that some endogenic compounds (e.g. uric acid,

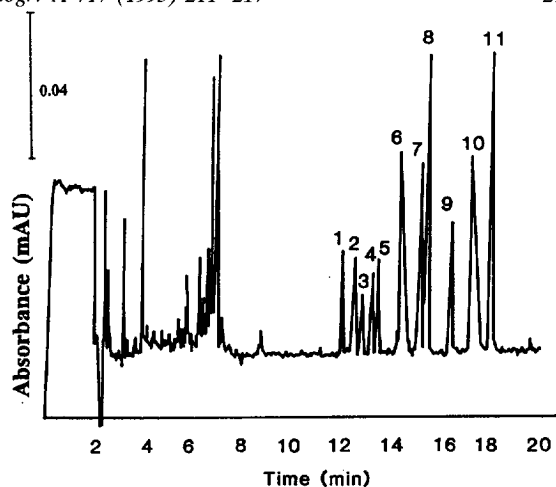


Fig. 4. Electropherogram of human urine spiked with 75 $\mu\text{g/ml}$ (except timolol and 2,6-dimethylphenol 150 $\mu\text{g/ml}$) of 1 = acebutolol, 2 = nadolol, 3 = timolol, 4 = atenolol, 5 = metoprolol, 6 = 2,6-dimethylphenol (internal standard), 7 = oxprenolol, 8 = pindolol, 9 = alprenolol, 10 = labetalol, 11 = propranolol. Migration conditions as described in the Experimental section. Capillary 68 cm \times 50 μm I.D.

Table 1

The IPC method. The resolution (R) and the number of effective theoretical plates (N) with standard deviations for β -blockers spiked in serum and urine

Compound/ matrices	R	N ($1/m$) \times 100
1 a ^a	6.2 ± 0.3	24 ± 2
b ^a	3.1 ± 0.1	49 ± 1
2 a	0.7 ± 0.2	47 ± 6
b	0.5 ± 0.1	69 ± 10
3 a	7.0 ± 0.7	52 ± 7
b	3.6 ± 0.4	62 ± 6
4 a	7.7 ± 0.4	67 ± 10
b	3.5 ± 0.3	67 ± 8
5 a	6.8 ± 0.9	289 ± 16
b	3.4 ± 0.4	378 ± 41
6 a	4.8 ± 0.6	448 ± 33
b	2.6 ± 0.4	409 ± 47
ISTD a		700 ± 75
ISTD b		654 ± 54

^a a is serum and b is urine. Number of replicates is 6 for both a and b.

Wavelength, 260 nm. Column, Asahipak C8P-50. Compounds: 1 = atenolol, 2 = acebutolol, 3 = metoprolol, 4 = oxprenolol, 5 = alprenolol and 6 = propranolol.

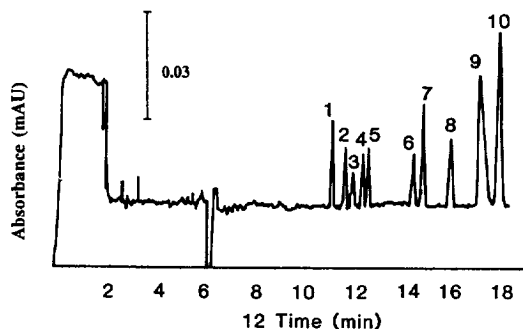


Fig. 3. Electropherogram of human serum spiked with 400 $\mu\text{g/ml}$ of 1 = acebutolol, 2 = nadolol, 3 = timolol, 4 = atenolol, 5 = metoprolol, 6 = oxprenolol, 7 = pindolol, 8 = alprenolol, 9 = labetalol, 10 = propranolol. Migration conditions as described in the Experimental section. Capillary 56 cm \times 50 μm I.D.

glucose conjugates) and exogenic compounds (e.g. caffeine and its metabolites) in urine may interfere with the screening of β -blockers, especially when the drugs are present at low concentrations (e.g. below $0.1 \mu\text{g/ml}$); thus IPC (see R -values in Table 1) is less reliable in urine than in serum, where the concentrations of the interfering compounds are lower. In MECC the reverse applies, with the difference in separation efficiencies in the two matrices being much smaller (Table 2). However, in the MECC system the resolution of the fifth compound (metoprolol) from oxprenolol is better in serum than in urine, because serum samples were analyzed without an internal standard [as the internal standard (2,6-dimethyl phenol) migrates

poorly]. In contrast to resolution values, in the IPC system the effective theoretical plate numbers are higher in urine than in serum samples. Exceptions were oxprenolol and propranolol (Table 1). From Table 2 the same phenomenon is seen in the MECC system. The only exception is labetalol, which gives N -values larger in serum than in urine matrix due to the improved recovery after the clean-up steps. According to Table 2, the hydrolyzation and the solid-phase extraction caused greater variation in the N -values calculated with labetalol than in those calculated with the other β -blockers. Furthermore, labetalol showed an exceptional behaviour for a β -blocker in our MECC studies with organic buffer modifiers [11].

By employing gradient elution in the IPC system, the interaction of other normally used β -blockers such as sotalol, nadolol, timolol, pindolol and labetalol could also be studied. The results showed that sotalol interfered with the analysis of atenolol (substantial co-elution). Nadolol was eluted together with caffeine, timolol with acebutolol, pindolol with oxprenolol and labetalol with ISTD. These compounds were then excluded from the IPC studies. In the MECC methods described here, neither of these parent compounds nor caffeine interfered with each other during the analyses, because the endogenic compounds and caffeine migrated before t_0 . Though the resolution values in IPC are larger than in MECC, all of these ten β -blockers could be separated in a single MECC run. Comparison between N -values obtained by IPC and MECC methods shows that in MECC early migrating β -blockers have approximately ten times more theoretical plates than in IPC. However, in IPC and in MECC slower-eluting compounds have equal N -values because of the gradient step in IPC. The Asahipak column used in this study has also been reported to give high effective plate numbers [17].

Table 2

The MECC method. The resolution (R) and the number of effective theoretical plates (N) with standard deviations for β -blockers spiked in serum and urine

Compound/ matrices	R	N ($1/m$) \times 100
1 a ^a	1.83 ± 0.18	196 ± 43.3
b ^a	2.50 ± 0.02	263 ± 7.60
2 a	1.10 ± 0.10	178 ± 41.0
b	1.22 ± 0.06	216 ± 2.65
3 a	1.32 ± 0.11	217 ± 50.1
b	2.00 ± 0.08	304 ± 66.0
4 a	0.96 ± 0.10	260 ± 47.6
b	1.26 ± 0.04	295 ± 4.11
5 a	5.84 ± 0.41	326 ± 58.6
b	3.76 ± 0.24	417 ± 18.2
6 a ISTD	–	–
b	2.49 ± 0.18	172 ± 6.79
7 a	1.08 ± 0.12	324 ± 46.8
b	1.35 ± 0.02	388 ± 15.8
8 a	3.16 ± 0.32	408 ± 43.8
b	4.27 ± 0.12	446 ± 3.29
9 a	2.95 ± 0.58	366 ± 61.7
b	2.56 ± 0.17	531 ± 27.1
10 a	1.02 ± 0.22	285 ± 19.0
b	1.94 ± 0.06	147 ± 6.73
11 a		322 ± 82.8
b		542 ± 8.35

^a a is extract from human serum and b is filtrate of human urine. Number of replicates for a is 6 and for b, 5.

Wavelength, 214 nm. Compounds: 1 = acebutolol, 2 = nadolol, 3 = timolol, 4 = atenolol, 5 = metoprolol, 6 = 2,6-dinitrophenol (ISTD), 7 = oxprenolol, 8 = pindolol, 9 = alprenolol, 10 = labetalol and 11 = propranolol.

4. Conclusions

In capillary zone electrophoresis (CZE), the number of plates for drug analytes is typically at

least 1.5 orders of magnitude greater than in isocratic HPLC [18]. In this study with MECC, we had approximately one order of magnitude more effective plates than in IPC for the β -blockers studied. In many applications the CZE or MECC system with higher resolution can be competitive with HPLC even after a great deal of efficiency is lost. This is particularly true in terms of instrumentation: CZE is simpler and easier to apply, and the basic instrument can be very inexpensive. In addition, there is a great difference in the cost of analyses, e.g. the volumes of solvents, buffers and samples. Furthermore, the clean-up steps of column materials after each IPC run in HPLC are time-consuming.

The resolution of the ten β -blockers was not good enough for simultaneous routine screening of the compounds in a single IPC run. However, using the MECC in this study, the ten β -blockers can be completely separated in a single run.

The main advantage of IPC over MECC noted in this study is that the detection by diode-array detector can be done at the desired wavelength, which also makes it possible to eliminate the absorbance of the background. Furthermore, IPC is more sensitive than MECC in analyzing β -blockers under the conditions described: in IPC the detection limits varied from 100 to 670 ng/ml in serum [2], and in MECC from 10 to 20 μ g/ml in urea [8] and 1 to 50 μ g/ml in serum [9]. Of the two methods, only IPC can be used for clinical bioanalyses of β -blockers, when parent compounds must be determined. The detector response in MECC is too low when unconcentrated samples are analyzed. However, when the real samples are concentrated about eight-fold, the native β -blockers in biological matrices may be detected with the UV technique.

The effectiveness of MECC can be seen in Tables 1 and 2. All ten drug compounds can be separated from each other in a single run in MECC, and the pretreatment steps of the biological samples (serum) are easier and simpler than those in IPC measurements. The diminutive sample preparation requirements present an attractive advantage. In addition, being able to

omit the extraction step results in greater accuracy. The main drawbacks with CZE involve peak identification and occasional difficulties with biological matrices. These disadvantages were surmounted by using MECC.

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