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Screening of /I-blockers in human serum by ion-pair chromatography and their identification as methyl or acetyl derivatives by gas chromatography-mass spectrometry

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ABSTRACT

A simultaneous screening method for atenolol, acebutolol, metoprolol, oxprenolol, alprenolol and propranolol by ion-pair chromatography with a column-switching technique was developed. The serum samples were purified using either liquid-liquid extraction or solid-phase extraction methods. The pretreatment of the samples consisted of hydrolysis and protein precipitation. The drug separation was on either octadecylsilica or polymer-based alkyl column material. Binary eluent mixtures containing methanol and a buffer solution with a quaternary ammonium salt as an ion-pair former were used. Detection of the compounds in liquid chromatographic analysis was based on ultraviolet spectra. The effects of methanol, two buffers and the ion-pair former on the retention of the compounds were studied. The determination limits ranged from nanograms to micrograms in the ion-pair chromatographic method, depending on the drug studied. Identification was based on the mass spectra or, if necessary, on selected-ion monitoring spectra of either the methylated or the acetylated compounds obtained by means of gas chromatographyelectron impact or negative chemical ionization mass spectrometry. The detection limits for the identified compounds were in the picogram range. The matrix effect was strong, and this resulted in determination limits in the nanogram range with the scan method.

INTRODUCTION

β-Adrenoceptor blocking drugs are of therapeutic value in the treatment of various cardiovascular disorders, such as angina pectoris, cardiac arrhythmia and hypertension. They are so sensitive that even a small oral dose of the drug gives sufficient blockade [1]. Because of the sedative effect of the P-blockers they are also misused as doping agents in some sports [2,3].

β-Blockers are exceptionally toxic and most of them possess a narrow therapeutic range; the difference between the lowest therapeutic and highest tolerable doses is small. Their unusually low concentrations in human blood makes their analysis difficult. In addition, the half-lives ($t_{1/2}$) of these drugs in serum or plasma are only a few hours, except for alprenolol [1]. They therefore require a sensitive and rapid screening method. Special problems can arise during screening of biological fluids, such as urine or serum, because low concentrations of the drugs or their metabolites have to be determined in these matrices, which contain high concentrations of other endogenous compounds or proteins.

Polar P-blockers have previously been analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) using ultraviolet (UV) or highly specific fluorescence detectors [4]. As separation of ionic compounds in RP-HPLC is based on their weakly acidic or basic functional groups, ion-pair chromatographic (IPC) methods can be used for analyses for these drugs by adding organic compounds to the eluent to form ion pairs with oppositely charged sample molecules. In analyses for

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these drugs either the negative or positive charge must be masked by the counter-ion and the other suppressed by choosing an appropriate pH. The presence of a basic amino group in an analyte often leads to long retention times. Control of the mobile phase pH and/or addition of amines to the mobile phase are frequently used cures for peak tailing [5,6].

Often liquid-liquid extraction (LLE) is used to clean up and separate drugs from the matrices. The choice of the solvent in LLE plays no significant role because all the usual organic solvent have been proved to be suitable. The pretreatment of the sample has sometimes included back-extraction of the drugs into the aqueous solution, which is considered to be a good procedure for cleaning up the organic material.

LLE has been found to be an inadequate clean-up procedure as far as very lipophilic compounds in plasma are concerned. Therefore, the use of solid-phase extraction (SPE) as a clean-up procedure has increased in analyses for both hydrophilic and hydrophobic compounds. Moreover, especially the recovery of P-blockers, like that of many other drugs, is better with SPE than LLE, which further expanded the use of the technique for their purification.

Gas chromatography-mass spectrometry (GC–MS) is both a primary and a confirmation technique in analyses for P-blockers [7]. The drugs are first extracted by LLE from urine before derivatization of their polar functional groups. In human doping analysis, and also in horse doping, blood samples may increase the reliability of the results if serum is to be used in the case where no good method for screening exists or more precision is demanded for reliable results.

In this paper we present a method for simultaneously screening the /&blockers atenolol, acebutolol, metoprolol. oxprenolol, alprenolol and propranolol in human serum by ion-pair chromatography with UV detection and their confirmed identification as their methyl and acetyl derivatives by GC-MS. Ion-pair chromatographic analyses were carried out using either acetate or phosphate buffers containing N-cetyl-N,N,N-trimethylammonium bromide (CTAB). The technique was based on ion-pair formation of the drugs with CTAB. After injection the compounds were adsorbed on the column material, and both the water-soluble and non-ion-pair-forming compounds were eluted using pure water as the mobile phase for 1 min to waste.

Using biphenylamine as an internal standard (I.S.), we found the method to be suitable for pharmacokinetic studies and investigated the possible interference of caffeine and five other /&blockers, namely sotalol, nadolol. timolol, pindolol and labetalol. The separation of the enantiomers of these pharmaceuticals was not studied owing to the selection of the column materials and to their unknown stabilities in the presence of the chiral compounds in the eluent.

Our main aim was to obtain information for micellar electrokinetic capillary chromatography (MECC) of the parent p-blockers using CTAB as the micelle and to have a reference technique for HPLC for the determination of these drugs in biological fluids [8]. Hence the metabolites were not screened by HPLC. In addition, the UV spectra of the ion-paired metabolites were not known. However, in identification by GC-MS the metabolites were registered and identified, because reference data were available in our system library and in the literature [9].

EXPERIMENTAL

Apparatus

Liquid chromatography. The liquid chromatograph was a Hewlett-Packard Model 1090 instrument equipped with a Model 1040A diode-array detector, a computer, a disc drive unit, an integrator, a printer and a plotter (Hewlett-Packard, Avondale, PA, USA). The columns used were Hypersil C_{18} (10 and 100 × 4.6 mm I.D., 5 μm), Shiseido SG-120 (polymer-based C_{18}) (150 × 4.6 I.D., 5 μm), HP Guard C_{18} (5 × 4.6 mm I.D., 5 μm), Hibar® LiChrosorb RP-18 (Merck) (250 × 4.6 mm I.D., 10 μm) and Asahipak C8P-50 (Asahipak) (150 × 4.6 mm I.D.. 5 μm). Detection was at 230, 260 and 280 nm with a IO-nm wavelength width. The reference wavelength was at 500 nm. After injection the compounds were adsorbed on the column material, and both the water-soluble and non-ion-pair-forming compounds were eluted to waste for l min by column switching using pure water as the mobile phase. The samples were separated at ambient temperature (22–26°C).

Gas chromatography-mass spectrometry. A Hewlett-Packard Model 5989A single-stage quadrupole mass spectrometer was used with electron impact (EI, 70 eV) or negative chemical ionization (NCI). A Hewlett-Packard Model 5890A gas chromatograph, an HP 98785A monitor, an HP 6000 330S digital data storage, an HP 9000 345 data system and an HP LaserJet III printer were used for analysis, data storage and reporting. The carrier gas (helium) was purified with a Supelco high-capacity carrier gas purifier (Supelco, Bellefonte, PA, USA).

The compounds were separated on an HP ULTRA-1 high-performance GC column (12.5 m × 0.20 mm I.D., 0.33 μm). The temperature was programmed from 125 to 310°C at 15 and 10°C/min. The temperatures of the injector, transfer line, source and quadrupole were 260, 280, 270 and 120°C, respectively. The carrier gas was helium (1 .0 or 1.5 ml/min at 150°C) and the CI reagent gas was methane. Injection was done by the solvent flush method (1 or 2 μl with solvent plug) with methanol–toluene (4:96, v/v) as solvent. The full-scan mass spectra of the /?-blockers were scanned from 40 to 650 u at a rate of 0.96 ms/u. When necessary selective ion-monitoring technique (SIM) was also used with at least six main fragments obtained from the scan spectra of the derivatized parent compounds.

Materials and reagents

The β-blockers used were acebutolol hydrochloride, alprenolol hydrochloride, atenolol, labetalol hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxprenolol hydrochloride, pindolol, (*S*)-(−)-propranolol hydrochloride, timolol maleate and anhydrous caffeine (Sigma, St. Louis, MO, USA).

The tablets taken by volunteers were 100 mg of alprenolol (Aptin N, Hassle), 25 mg of propranolol (Propral, Medipolar), 40 mg of oxprenolol (Trasicor, Ciba), 100 mg of acebutolol (Espesil, Orion), 50 mg of metoprolol (Seloken, Astra) and 50 mg of atenolol (Tenoblock, Leiras). The doses were given once to the volunteers so that another drug was not taken before the urine taken from the volunteer was equal to the drug-free urine. The P-blockers were administered after overnight fasting. Serum samples were separated by centrifugation and stored frozen in PTFE tubes until analysed.

The high-purity solvents and analytical-reagent grade reagents were as follows: glycine, NaOH, CH_2Cl_2, propanol, methanol (LiChrosolv), Na_2SO_4, H_2SO_4, zinc sulphate, diethyl ether, glacial acetic acid, sodium acetate, NaH_2PO_4, Na_2HPO_4, pyridine, biphenylamine, N-cetyl-N,N,N-trimethylamine bromide (CTAB), potassium carbonate, acetic anhydride, methyl iodide, potassium hydrogenphthalate and disodium tetraborate decahydrate (Merck, Darmstadt, Germany), acetone and toluene (Rathburn, glass distilled) and N,N-dimethyloctylamine (DMOA, 95%, Aldrich) and distilled, ionized water (Water I-system, Gelman Sciences, Ann Arbor, MI, USA). β-Glucuronidase (EC 3.2.1.31) Type H-l from *Helix pomatia* (*416 800* units/g), stored at − 20°C (Sigma), was used for enzymatic hydrolysis.

The pH of the buffer solutions used in mobile phase was adjusted using a Jenway 3030 pH meter and electrode (Jenway, Felsted, UK) containing 4 A4 KC1 in saturated AgCl. Calibration was made with potassium dihydrogenphthalate (0.050 *M*, pH 4.00) and sodium tetraborate (0.010 *M*, pH 9.81) buffer solutions.

Ultrasonication was performed with Eurosonic 44 (Oriola, Prolab). The blood samples were centrifuged at 1000 g with a Heraus Christ Medifuge. Sartorius Minisart NML sterile filter units (0.45 μm; Sartorius, Göttingen, Germany) and Millex filters of 0.5-μm pore size from Millipore (Nihon Millipore, Yonezawa, Japan) were used for filtration of the samples. The eluents containing CTAB were filtered through 0.45-μm membranes (Millipore, Mosheim, France) and degassed with helium before use.

Preparation of standard solutions

Stock standard solutions of the respective drugs (1 mg/ml) were prepared in methanol. Calibration standards for biological fluids were made by adding each standard solution to the blank biological medium.

Pretreatment of blood

The respective P-blockers were administered to two volunteers who were coffee or tea drinkers, non-smokers or non-drug-users in the morning after overnight fasting. Blood samples from the volunteers were collected in 5- and 10-ml VT-050PZX Venoject tubes with a silicone coating (Terumo Europe, Belgium) 2 and 3 h after administration of the

drugs. They were kept at ambient temperature (22–26°C) for 1 h, after which they were centrifuged for 10 min at 1000 g (3000 rpm). The incubation of the serum samples was carried out with Type H-l enzymes at 60°C for 1 h or overnight at ambient temperature.

Spiked serum sample preparation

The MIX6 sample solution, which was a mixture of six β-blockers, was made by adding 500 μl of each stock solution (see *Preparation of standard solutions)* to *900* μl of serum [hydrolysed, proteins precipitated and eluted through Supelclean LC-18 3 ml-SPE tubes (Supelco)]. The working concentration was 2.56 μg in 20 μl (loop volume *20* μl).

Pretreatment of the serum samples by liquid-liquid extraction

Method I. A 50-μl volume of the IS. (1 mg/ml), 50 μl of glycine buffer (0.5 *M*, pH 12), 50 μl of NaOH (2 *M*), 1 ml of saturated NaCl solution and 4 ml of mixture containing CH_2Cl_2 and propanol (3:97, v/v) were mixed with 3 ml of serum. The mixture was shaken mechanically for 10 min. After centrifugation for 5 min at 2000 g, the organic layer was separated, dried with Na_2SO_4 for 10 min and further centrifuged for 5 min. The organic phase was then filtered and analysed by LC [10].

Method II. A 50-μl volume of the I.S.. 0.1 ml of NaOH (0.2 *M*) and 6 ml of CH_2Cl_2 were mixed with 3 ml of serum. The mixture was shaken mechanically for 10 min and centrifuged for 10 min at 2000 g. The organic layer was then separated and added to 0.1 ml of sulphuric acid (0.025 *M*). After shaking for 1 min and centrifugation for 5 min, the organic phase was separated and analysed by LC [11].

Method III. A 50-μl volume of the I.S. (1 mg/ml), 0.5 ml of zinc sulphate (0.70 *M*), 0.5 ml of DMOA (0.5 m*M*) and 0.5 ml of NaOH (1 .O *M*) were mixed with 3 ml of serum, shaken mechanically and centrifuged. The water phase was filtered and analysed by LC [12].

Method IV. A 50-μl volume of the I.S., 0.5 ml of NaOH (5 *M*), 2 ml of diethyl ether and 3 g of Na_2SO_4 were mixed with 3 ml of serum. The mixture was shaken mechanically for 10 min and centrifuged for 5 min. The diethyl ether layer was separated and evaporated off. The residue was dissolved in 2 ml of methanol-water (I: 1, v/v) and analysed by LC.

Solid-phase extraction

SPE columns were regenerated with methanol and distilled, deionized water. The samples were adsorbed on the C_{18} phase, washed with 1 ml of water, dried in vacuum and eluted with 2 ml of water–methanol (10:90, v/v). In screening runs the sample volume was 2 ml, but in quantitative studies the volume was reduced to 600 μl by evaporation of the solvent.

Preparation of the buffers

The buffers used in LC separation were made from 0.1 *M* sodium acetate by decreasing the pH to 6.0 with both 50% and 1% glacial acetic acid, and 0.1 A4 sodium dihydrogenphosphate and 0.1 *M* disodium hydrogenphosphate (pH 7.0). After adjusting the pH to 6.0 or 7.0, 9 m*M* of N-cetyl-N,N,N-trimethylammonium bromide were added into the buffer. The pH values of the solutions were not changed by the addition.

HPLC gradients

The /I-blockers were eluted with two solvent gradient methods. With the acetate buffer the methanol gradient was increased from 4% at rate of 6% per 2 min to 10%, at 20% per 4 min to 30% and finally at 30% per 6 min to 60%. When the LC separation was made using phosphate buffer, the methanol gradient started from 30%, which was maintained for 4 min. It was then increased at a rate of 5% per 4 min to 50% and finally at 10% per 2 min to 60%.

Derivatization for GC-MS

Methylation. After the SPE treatment the methanol extract was evaporated in a heating block under nitrogen. A 1.5-g amount of K_2CO_3, 200 μl of acetone and 50 μl of CH_3I were added to the dry residue. The resulting solution was incubated at 60°C for 60 min before the acetone was evaporated under nitrogen, after which the residue was dissolved in 100 μl of methanol-toluene (4:96, v/v). GC-MS was carried out on the derivatives contained in the methanolltoluene mixture by injecting 2 μl of the solution.

Acetylation. After the SPE clean-up the extract

was evaporated under nitrogen as described in the methylation process. The residue was dissolved in 150 μl of acetic anhydride-pyridine (2:3, v/v) and incubated at 80°C for 60 min before being analysed by GC-MS.

RESULTS

Chromatographic separation in LC

In the absence of the ion-pair reagent, CTAB, the compounds were eluted in one zone without peak

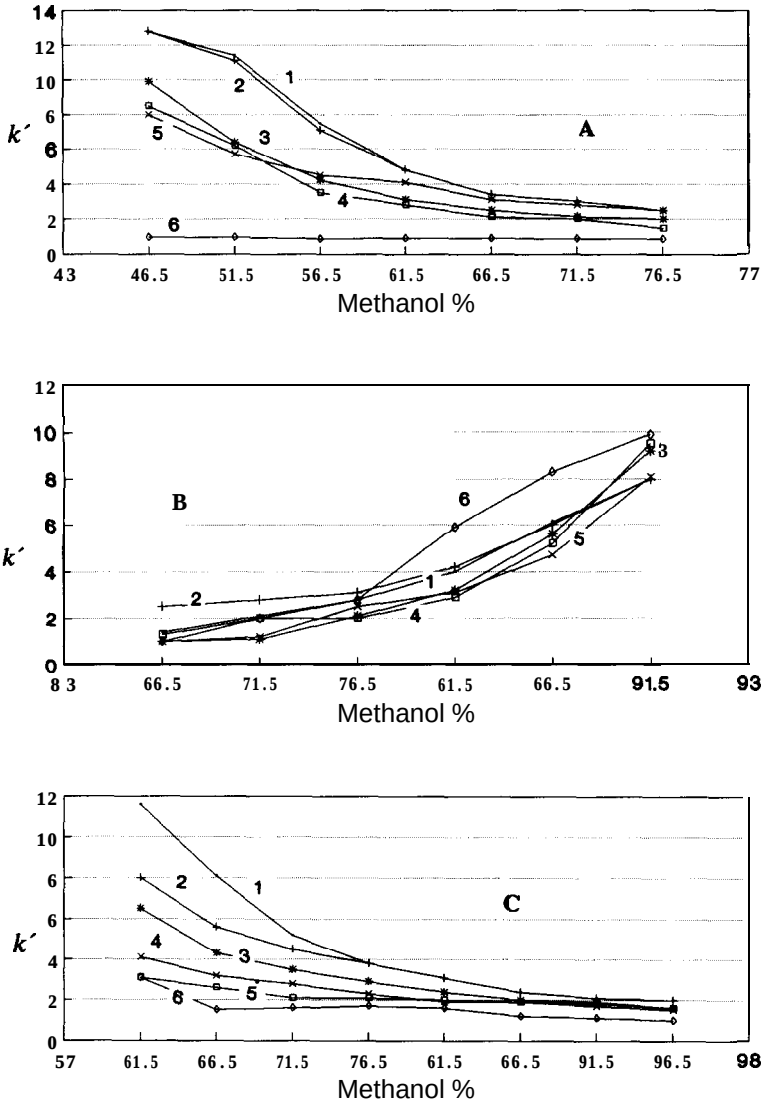

Fig. 1. Effect of methanol content on the retention of /l-blockers in ion-pair chromatography. (1) Alprenolol, (2) propranolol, (3) oxprenolol, (4) acebutolol, (5) metoprolol and (6) atenolol. UV detection at 230 nm. Column, Shiseido SG-120; ion-pair former, CTAB. Mobile phases: (A) phosphate buffer without CTAB; (B) CTAB without buffer; (C) acetate buffer without CTAB. Flow-rate 0.7 ml/min.

TABLE I

RELATIVE RETENTION TIMES OF THE COMPOUNDS STUDIED

LC conditions: Asahipak C8P-50 column; eluent; acetate buffer containing CTAB and methanol: gradient elution; detection at 260 nm. CC-MS conditions as described under Experimental. Flow-rate of the carrier gas. 1.5 ml/min.

Compound	Relative retention time		
	HPLC, ion-paired drugs	GC–MS, methylated drugs	GC-MS, acetylated drugs
Atenolol	0.205	2.97	3.02
Sotalol	0.21 I		–
Caffeine	0.377	1.51	I.43
Nadolol	0.395	2.88	
Metoprolol	0.527	2.56	3.04
Timolol	0.562	2.86	–
Acebutolol	0.608	3.84	2.90
Pindolol	0.665	2.71	–
Oxprenolol	0.692	I.93	2.71
Alprenolol	0.926	1.74	2.69
Propranolol	0.854	2.51	3.21
Labetalol	0.976	4.26	–
Biphenylamine (I.S.)	1.00	1.00	1.00

resolution, making it impossible to analyse for all six /I-blockers simultaneously. It was known that the members of the b-blocker group were difficult to separate under reversed-phase conditions on modified phases in the pH range 1.5–7.5 [13]. Therefore, we had to optimize the chromatographic conditions. The eluent composition has a clear effect on the resolution of the ion pairs, as can be seen in Fig. 1 and in the chromatograms in Figs. 2 and 3. Fig. 1 shows the capacity factors of the P-blockers to de-

TABLE II

NUMBER OF EFFECTIVE THEORETICAL PLATES, k', α AND R FOR P-BLOCKERS SPIKED IN SERUM

λ = 260 nm. Column: Asahipak C8P-50. Gradient elution (see Experimental).

Compound[a]	Matrix[b]	k'	N (plates per metre)	α	R[c]
I	a	4.76 ± 0.23	3800 ± 800	2.85 ± 0.06	13.42 ± 0.71
	b	4.24 ± 0.09	4900 ± 100	I.91 ± 0.03	3.10 ± 0.15
2	a	13.58 ± 0.53	9700 ± 800	I.15 ± 0.01	2.96 ± 0.40
3	b	8.38 ± 0.27	6900 ± 1000	1.07 ± 0.03	0.51 ± 0.12
3	a	15.66 ± 0.46	2000 ± 2600	I.14 ± 0.00	3.2X ± 0.19
2	b	8.43 ± 0.15	6200 ± 600	I.76 ± 0.09	3.62 ± 0.41
4	a	17.89 ± 0.52	24 000 ± 2000	1.24 ± 0.01	6.88 ± 0.28
	b	14.89 ± 0.51	6700 ± 800	1.50 ± 0.04	3.51 ± 0.29
5	a	22.14 ± 0.61	57 000 ± 13 800	1.09 ± 0.01	3.51 ± 0.35
6	b	21.78 ± 0.20	37 800 ± 4100	1.25 ± 0.02	6.79 ± 0.85
6	a	24.10 ± 0.74	68 800 ± 10 000	1.07 ± 0.02	3.27 ± 0.35
5	b	27.96 ± 0.30	40 900 ± 4700	1.07 ± 0.01	1.39 ± 0.12
I.S.	a	25.73 ± 1.04	69 600 ± 7600		
	b	28.72 ± 0.78	65 400 ± 5400		

[a] Compounds: 1 = atenolol; 2 = metoprolol; 3 = acebutolol; 4 = oxprenolol; 5 = propranolol: 6 = alprenolol.
[b] a = Acetate buffer eluent; b = phosphate buffer eluent.
[c] R = Resolution.

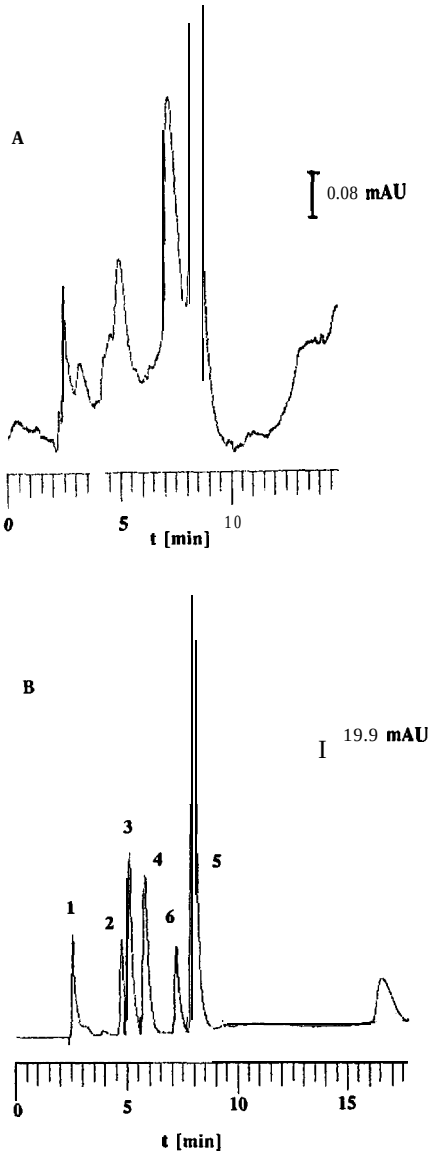

Fig. 2. HPLC of the separation of /?-blockers with the ion-pair technique. (A) Blank human serum sample; (B) with six j-blockers added to human serum sample. Gradient elution (see Experimental). Compounds: 1 = atenolol; 2 = acebutolol; 3 = metoprolol; 4 = oxprenolol; 5 = propranolol; 6 = alprenolol. Eluent, phosphate buffer-methanol containing 9 m*M* CTAB; detection wavelength, 260 nm.

crease with increasing methanol concentration of the eluent when CTAB was not used. However, when no buffers were used, ***i.e.,*** phosphate or acetate ions were not present, the capacity factors increased with increasing methanol concentration. When both the buffer and the ion-pair former were in the mobile phase the use of methanol led to the same trend as in buffer solutions of pH 6.0 and 7.0.

Biphenylamine was chosen as the internal standard, because it eluted at the end of the drug zone and therefore did not interfere with the separation of the /?-blockers. The elution order of all the drugs analysed when the acetate buffer with CTAB and methanol was used is given in Table I. The hydrophilic compounds were eluted first: atenolol, acebutolol, metoprolol, oxprenolol, alprenolol and propranolol. The elution order of acebutolol and metoprolol and also that of alprenolol and propranolol changed and the pressure in the HPLC system increased considerably when the acetate buffer was changed to phosphate. The number of effective theoretical plates for the β-blockers spiked in serum were higher with the acetate than the phosphate buffer (Table II). As can be seen from the standard deviations, the methanol gradient elution is a valid method to separate these six &blockers from each other.

According to this study, the best column materials for the separation of the six /?-blockers were Hibar C_{18} and Asahipak C8P-50 materials. Fig. 2 shows chromatograms obtained from the SPE-treated blank and with P-blocker-spiked human serum samples. The peaks eluted within 10 min because of the methanol gradient. Fig. 3 shows the liquid chromatograms obtained from the real serum samples taken from the volunteers. Fig. 3A–C show that baseline resolution is not achieved when small drug concentrations are to be determined. However, with normally treated patents the parent compounds can be easily identified from profile chromatograms (Fig. 3D and E) 3 h after oral administration.

It became evident that all the extraction methods were tedious and lacked high and reproducible absolute extraction efficiencies. Table III shows the recovery ratios obtained with the LLE techniques (I-IV) using the two eluent buffers. The recovery ratios of propranolol were very poor for each method and could not be improved by changing the buffer. These results suggest that selective extraction eluents should be used for each drug. In contrast to LLE, SPE gave high recovery ratios in all instances. Therefore, we preferred SPE to LLE in further studies.

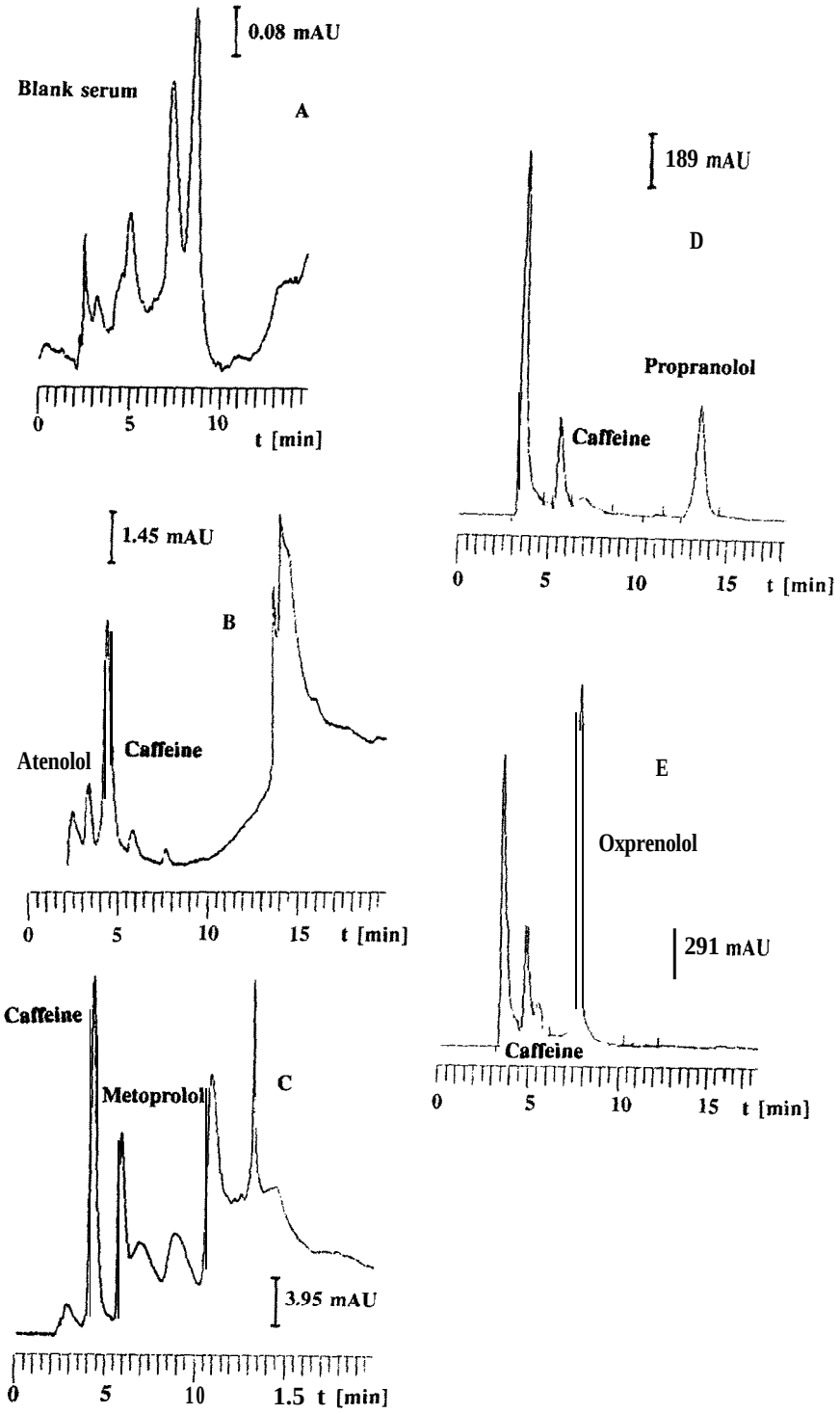

Fig. 3. Liquid chromatograms obtained from (A) blank serum and from I-ml human serum samples taken 3 h after administration of (B) atenolol and (C) metoprolol and from 3-ml serum samples taken 3 h after administration of (D) propranolol and (E) oxprenolol. Column, Asahipak C8P-50. Gradient Row with methanol (1 ml/min). Conditions as in Table 1.

TABLE III

RECOVERIES WITH LLE ONLY USING BUFFER AND METHANOL IN THE ELUENT

The recoveries were calculated against the internal standard, which was added to the matrix before SPE clean-up. Eluents in HPLC separation were without CTAB: (a) acetate buffer and methanol and (b) phosphate buffer and methanol. Column, Hypersil C_{18}.

Compound	Eluent	I	II	III	IV	SPE
Propranolol	a	7	11	15	8	78
	b	9	14	15	5	52
Metoprolol	a	12	7	4	21	86
	b	78	–	11	86	63
Oxprenolol	a	11	47	39	68	81
	b	16	–	9	86	76
Acebutolol	a	100	–	II	43	65
	b	50	–	10	27	67
Alprenolol	a	47	100	9	33	65
	b	18	–	10	68	66
Atenolol	a	–	100	33	100	87
	b	21	89	–	98	81

The liquid chromatograms of real serum samples showed that the concentrations of the β-blockers after oral administration are very low in human serum. Although possible co-eluting and interfering compounds could be removed from the biological fluid, the volume of the samples had to be fairly large (l-3 ml) when no preconcentration was done. Fig. 3 shows the chromatograms obtained from l- and 3-ml serum samples 3 h after oral administration of the tablet (see Experimental).

Accuracy and reproducibility in LC

The accuracy and reproducibility of the results for drug-free serum samples spiked with the /?-blockers were determined by comparing the peak-height ratios of 128 μg/ml of the drugs to the I.S. with those obtained for aqueous solutions containing similar concentrations of drugs and the IS.

The sensitivity limits of the drugs at 260 nm are given in Table IV. The calibration graph was obtained by plotting the ratio of the peak area of the drug to that of the I.S. The linearity of the plot of concentration of the drugs ***versus*** peak area from the detection limit (signal-to-noise ratio = 3) to 128.2 μg/ml in serum was tested. The results are given in Table IV. Calibration was carried out using the MIX6-spiked serum sample.

GC-MS

A poor GC response of /I-blockers, attributed to the interaction of hydroxy and amine groups with the column materials, was observed. Derivatization was carried out to improve their GC analyses. Fur-

TABLE IV

DETERMINATION LIMITS AND LINEARITY OF THE ION-PAIR CHROMATOGRAPHIC METHOD FROM DETERMINATION LIMIT to 128.2 μg/ml.

Eluent, acetate-methanol gradient (see Experimental); detection at 260 nm; ion-pair former, CTAB; column, Asahipak C8P-50. The concentrations of the /I-blockers in the determined linear range were the determination limits, 27.8, 47.6, 83.3 and 128.2 μg/ml. r is the correlation coefficient. The equation for the straight line was $y = bx + a$, where a is the intercept of the ordinate and b is the slope. The elution order was as in Table I.

Compound	Determination limit[a] (ng/ml)	a^b	b^b	r^b
Atenolol	630	12.58/9.59	5.08/0.27	0.982/0.987
Metoprolol	580	24.19/23.00	4.93/0.20	0.987/0.994
Acebutolol	500	23.13/24.57	4.22/0.21	0.992/0.991
Oxprenolol	350	21.27/22.40	2.64/0.12	0.997/0.997
Alprenolol	670	20.66/23.30	3.65/0.19	0.998/0.999
Propranolol	100	18.60/23.10	0.71/0.03	0.993/0.997

[a] Signal-to-noise ratio = 3.
[b] First values calculated from peak heights; second values calculated from peak areas.

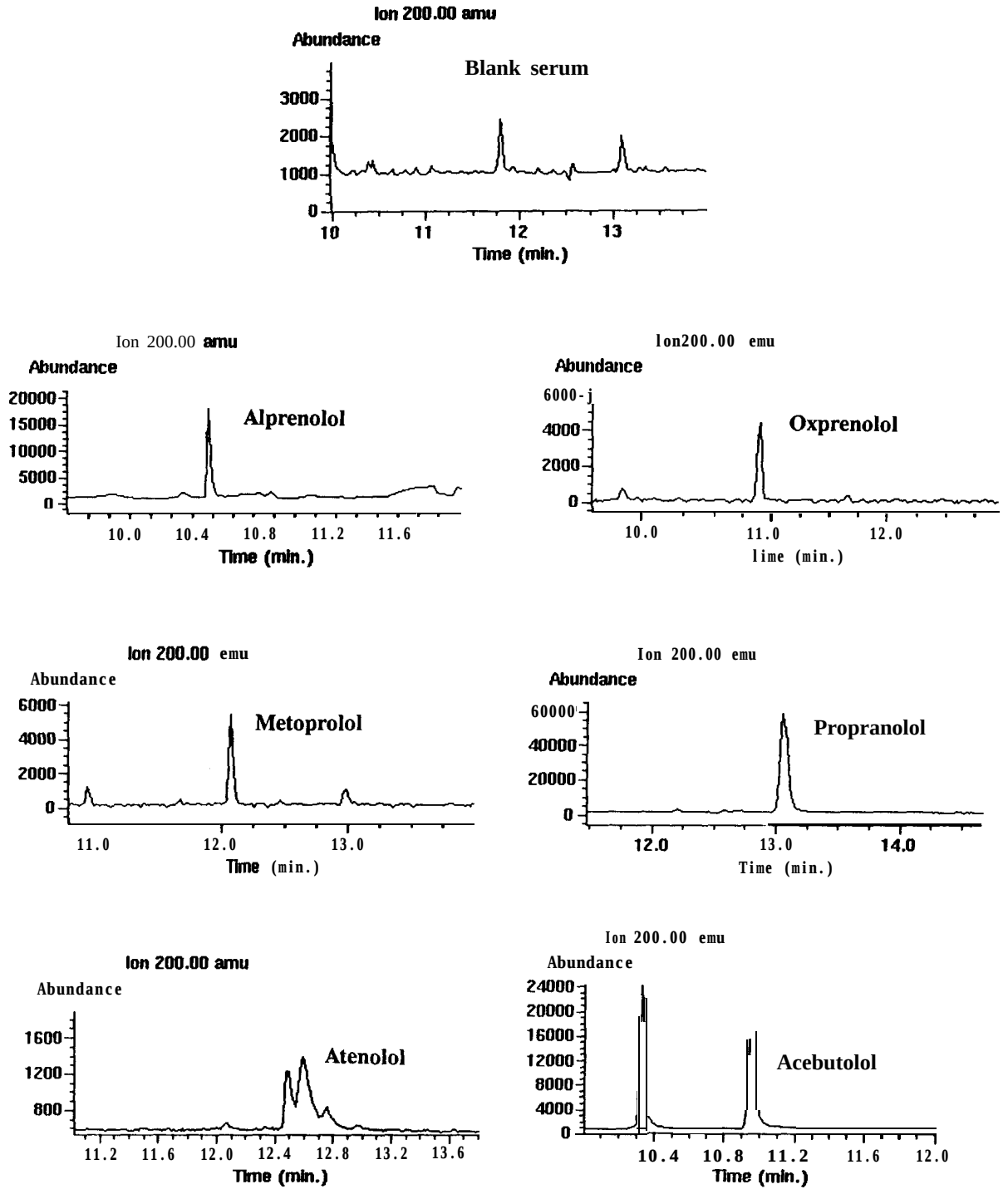

Fig. 4. Selected ion chromatograms of acetylated serum samples obtained by CC-El-MS. Conditions as described under Experimental

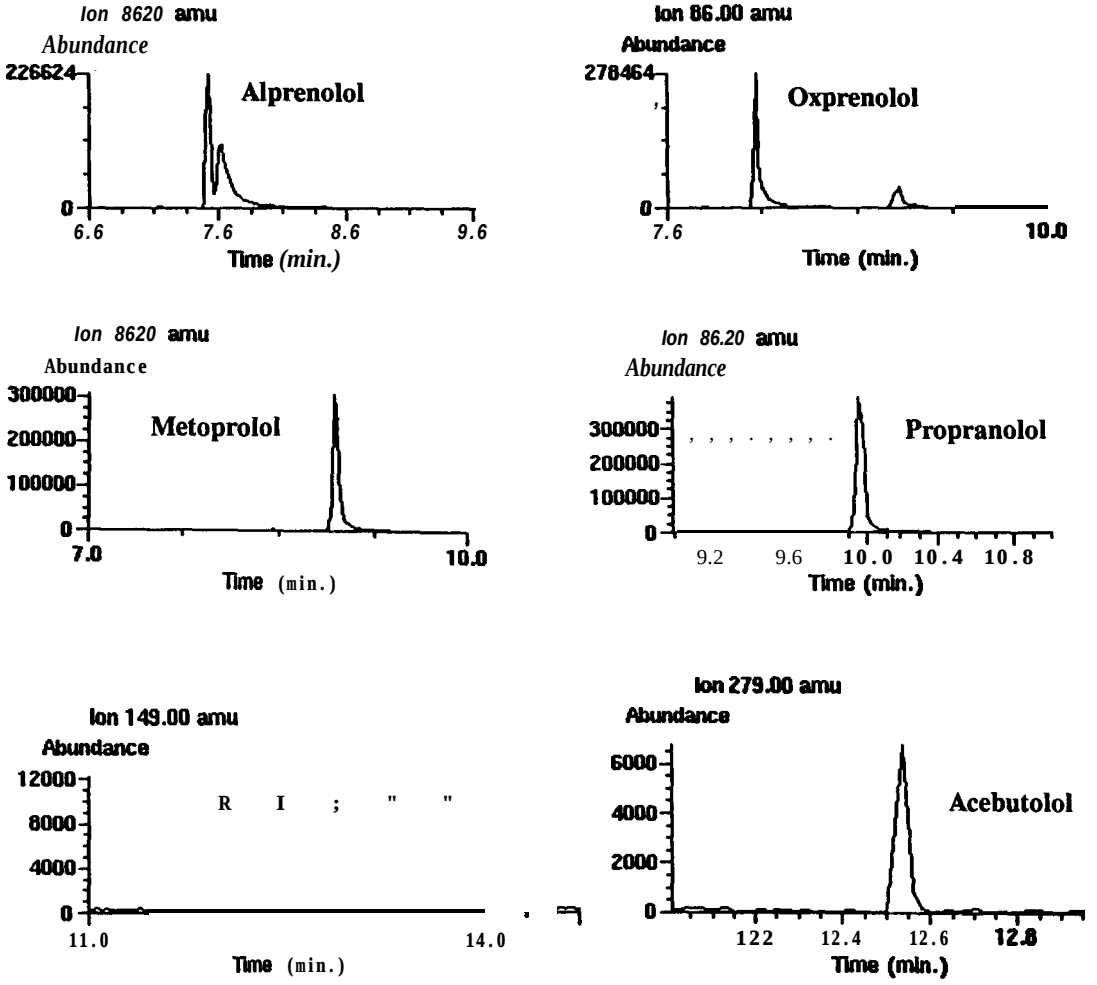

Fig. 5. Selected ion chromatograms of methylated serum samples obtained by GC-negative-ion-CI-MS. Run conditions as described under Experimental.

ther, typical fragments from derivatized oxypropanolamine chain help in the identification of the compounds in the overall mass spectra.

All the methylated and acetylated /?-blockers in the serum samples from volunteers who had taken the pharmaceuticals were identified. Identification of the acetylated and methylated /?-blockers was not complicated because only one product resulted from the incubation. The retention times of the derivatized β-blockers relative to the I.S. are given in Table I.

The most intense fragment ions observed from the methylated P-blockers were at ***m/z*** 149, 167, 71, 86, 91 and 107 and from the acetylated oxypropanolamine chain at m/z 72, 98, 140, 158 and 200. Lower detection limits were obtained by methylation than by acetylation in with alprenolol, atenolol, metoprolol and oxprenolol. In addition, the detection limits for acebutolol and propranolol were very low when they were acetylated and subjected to GC-EI-MS. The selective ion chromatograms of acetyl derivatives of the drugs are shown in Fig. 4, where they are compared with the chromatogram of the blank serum sample and those of the methyl derivatives in Fig. 5. Tables IV and V show the detection and determination limits for the studied /&blockers. The concentrations of the drugs in the serum samples taken from the volunteers are given in Table VI.

TABLE V

DETECTION LIMITS FOR THE β-BLOCKERS IN GC-MS ANALYSES

Data taken from SIM chromatogram (ions as in Figs. 4 and 5) obtained from scan analyses. Detection limits (LOD) calculated from the equation LOD = (V_{inj} 3 %E)/(peak–peak S/N), where %E = [(peak area of extracted drug in serum sample)/(peak area of non-extracted drug)]. V_{extr} *100%*. V_{extr} = extracted volume. Recoveries were determined from the mean of six replicates taken from each drug. S/N = signal-to-noise ratio.

Compound	Detection limit (ng)	
	Acetyl derivative	Methyl derivative
Acebutolol	26 (EI)	127 (EI)
	42 (NCI)	615 (NCI)
Alprenolol	124 (EI)	25 (EI)
	103 (NCI)	36 (NCI)
Atenolol	III (El)	81 (EI)
	105 (NCI)	67 (NCI)
Metoprolol	21 (EI)	3.1 (EI
	50 (NCI)	27 (NCI)
Oxprenolol	58 (EI)	51 (EI)
	34 (NCI)	32 (NCI)
Propranolol	21 (EI)	87 (EI)
	37 (NCI)	56 (NCI)

DISCUSSION

The ion-pair chromatographic screening and GC-MS identification methods described are useful for the determination of atenolol, acebutolol, metoprolol, oxprenolol, alprenolol and propranolol. However, the main metabolites were not screened in HPLC, because the UV spectra of the ion-paired compounds were not known. In addition, the clean-up techniques were not optimized for their extraction. Further, in SPE clean-up we should have used another ion-pair former compound in order to obtain better recoveries.

Details of the partition behaviour of drugs in extraction experiments are usually not given. Depending on the drugs being studied, it is possible to obtain high recovery ratios, accuracy and reproducibility by suitable adjustment and estimation of extraction parameters such as the solvent, volume ratio of the phases and the concentration of the base versus the ion pair.

The required pretreatment of the sample depends largely on the selectivity of the detection. Clean-up procedures such as back-extraction or extraction from a basic solution may be needed with less selective detectors. The use of troublesome or time-consuming clean-up methods considerably decreases the number of analyses that can be made in a laboratory. In addition, transfer of the extracts, evaporation steps and all other measurements may cause adsorption or losses of the sample. One way to overcome these problems is to use the column-switching technique in HPLC, where the dilute sample is injected into a precolumn. After washing with water the sample is easily transferred into the analytical column with the eluent. /I&Blockers are usually eluted quantitatively. By optimizing the HPLC eluent conditions the parent compounds could be eluted within 10 min. The HPLC method permits analyses of six β-blockers in a single serum extract

TABLE VI

DETERMINATION LIMITS FOR REAL SAMPLES AND CONCENTRATIONS OF DRUGS IN SERUM 3 h AFTER ADMINISTRATION

GC-EI-MS studies.

Compound	Determination limit (ng)		Concentration of drug in serum (ng/ml)
	Acetyl derivative	Methyl derivative	
Acebutolol	1.8	7.1	780
Alprenolol	0.6	7.6	120
Atenolol	3.2	5.3	330
Metoprolol	6.3	7.3	230
Oxprenolol	3.0	3.2	290
Propranolol	1.5	5.4	350

with high specificity. The retention order of the compounds was changed when phosphate was changed to acetate buffer and the pH was decreased.

The separation of the enantiomers of the pharmaceuticals was not studied owing to the separation of the column material and to the unknown stabilities in the presence of chiral compounds in the mobile phase during the gradient elution.

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