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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ANALYSIS OF THE ARYLOXYPROPANOLAMINES PROPRANOLOL, METOPROLOL AND ATENOLOL IN PLASMA AND TISSUE

HORST WINKLER, WALTER RIED and BJÖRN LEMMER*

Centre of Pharmacology, J.W. Goethe-University, Frankfurt/M. (G.F.R.)

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SUMMARY

A simple and rapid high-performance liquid chromatographic method is described for the quantitative analysis of three β-receptor blocking drugs of similar molecular structure (aryloxypropanolamines, AOPAs) but with different polarities. The method consists of extraction, reversed-phase ion-pair chromatography and fluorometric detection, whereby slight modifications in these parameters allow analysis of the different AOPAs in a similar way. The method was used to determine concentrations of propranolol, metoprolol and atenolol in plasma and various organs of the rat. In samples of 1 ml of plasma and 0.5—1.7 g of tissue, drug concentrations of at least 2 ng/ml and 5 ng/g, respectively, can be measured. Extension of the method to other AOPAs is possible.

INTRODUCTION

β-Adrenoceptor blocking drugs are of therapeutic value in the treatment of various cardiovascular disorders, such as angina pectoris, cardiac arrhythmia, hypertension (for review see refs. 1 and 2). A great number of β-blockers are available, which, however, differ not only in their specific β-adrenoceptor blocking effects (i.e. receptor affinity and selectivity, intrinsic sympathomimetic activity), but also in their non-specific effects. The latter can be related to the physicochemical nature of these drugs and can result in a membrane-stabilizing effect, a local anaesthetic action as well as a cardiodepressant effect [3—5].

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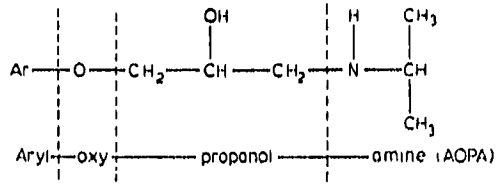
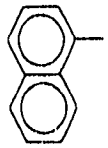
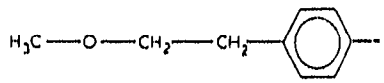
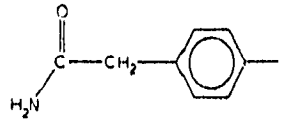
TABLE I

THE MOLECULAR STRUCTURE AND PARTITION COEFFICIENTS OF THE ARYLOXYPROPANOLAMINES PROPRANOLOL, METOPROLOL AND ATENOLOL



	Propranolol	Metoprolol	Atenolol
Ar =			
Partition coefficient (*n*-octane/phosphate buffer, pH 7.0, 20°C)	5.38	0.18	0.003

Most of the β-adrenoceptor blocking drugs in use are aryloxypropanolamines (AOPAs). Representative compounds of this group are propranolol, a highly lipophilic drug, atenolol, which is a highly hydrophilic compound, and metoprolol, a substance with an intermediate polarity (Table I).

A great number of methods have been described to determine the concentrations of these compounds, mainly in plasma or urine. These methods can be classified according to two analytical systems. Firstly, a spectrofluorometric system without chromatography was reported for propranolol [6—9] and atenolol [10, 11]. Secondly, different chromatographic systems have been described: gas chromatography has been used for propranolol [12], atenolol [13, 14] and metoprolol [15], thin-layer chromatography was described for propranolol [16] and atenolol [17], and, finally, high-performance liquid chromatography (HPLC) with spectrofluorometric detection was reported for propranolol [18—25] and atenolol [26—28].

Just recently, an HPLC method was published, which allows the detection of several β-blockers in plasma and urine; tissue samples were not studied [29]. Since β-adrenoceptor blocking drugs exert their pharmacological effects at the level of various organs, such as heart, lung and brain, drug concentration in these target organs are of utmost interest when studying the dynamic and kinetic behaviour of these compounds. In addition, tissue concentrations of β-blockers are also of importance in veterinary medicine.

The method presented in this paper takes advantage of the common molecular structure (AOPA) of the β-blockers, which differ in polarity because of the different aryl groups (Table I). This approach may be fruitful, since, as already mentioned, many different β-blockers are used therapeutically, of which, however, their concentrations can not be analyzed by a single method. In the following, an HPLC method with spectrofluorometric detection is described, which allows the detection and quantitation of β-blockers of different lipophilicity in plasma and various tissues of the rat. On the basis of their different polarities, the β-blockers propranolol, metoprolol and atenolol were chosen as representative compounds. Neither urine samples nor metabolites were included in the study, since only atenolol undergoes renal elimination, whereas propranolol and metoprolol are metabolized by the liver. Furthermore, the only metabolite of interest, 4-hydroxypropranolol, does not contribute to the pharmacological effect after intravenous administration of propranolol.

EXPERIMENTAL

Standards and reagents

All reagents used were reagent grade and purchased from Merck (Darmstadt, G.F.R.). Only deionized glass-distilled water was used. The racemic mixtures of propranolol hydrochloride and atenolol were kindly supplied by Rhein-Pharma (Planckstadt, G.F.R.), (±)-Metoprolol hydrochloride was from Hässle (Mölndal, Sweden).

Standard solutions from 2 mg/ml to 10 ng/ml were obtained from aqueous stock solutions (2 mg/ml) of each compound. These solutions were stored at 4°C for up to one month.

The mobile phase was prepared as follows. Ten millilitres of 85% phosphoric acid and 0.0025 moles of an alkyl sulfuric acid as sodium salt were mixed with 990 ml of water and with 990 ml of methanol, respectively. Both phases were filtered through a membrane filter (0.45 μm) and stored at 4°C. Before use the aqueous and methanolic phases were mixed as needed and degassed in an ultrasonic bath under vacuum.

HPLC instrumentation and conditions

The HPLC system consisted of a constant-flow pump (Gynkotek, 600/200), an injection valve (Waters, UK 6), a reversed-phase column (Knauer, 25 cm × 4.6 mm; LiChrosorb C_{18} 10 μm), a spectrofluorometer with a 150 W xenon lamp (Kontron, SFM 22), an absorbance detector (Waters, 440) and a computing integrator (Spectra Physics, SP 4100).

The mobile phase, which consisted of a methanol—water mixture containing alkyl sodium sulfate as counter-ion and 1.3% phosphoric acid to ionize the amine function of the compounds, was modified in regard to the polarities. The methanol—water ratio was 65:35 for propranolol and 60:40 for metoprolol and atenolol. As counter-ion octyl sodium sulfate was added for propranolol and metoprolol and dodecyl sodium sulfate for atenolol. The flow-rate was 2 ml/min. The activation monochromator of the fluorometer was set at 280 nm for all three compounds and the emission monochromator was set at 300 nm for metoprolol and atenolol and at 333 nm for propranolol. The fluorescence detector was connected to the integrator, and peak heights were measured. The flow system was controlled by the absorbance detector. Chromatography was carried out at ambient temperature.

Sample preparation

Plasma and tissue samples of various organs from light—dark-synchronized male Wistar rats of about 150—180 g were used [30]. The rats were sacrificed by decapitation, and blood was collected in 12-ml conical glass tubes containing 50 μl of heparin (250 I.U.). After centrifugation (900 *g*, 15 min) 1-ml plasma portions were pipetted into 12-ml screw-capped glass tubes and kept at −35°C. The organs were dissected out, rinsed in ice-cold 0.9% saline solution, blotted on filter paper, weighed, frozen in liquid nitrogen and finally stored at −35°C.

Plasma extraction (Fig. 1)

The 1-ml plasma samples were thawed and 0.5 ml of 2 *N* NaOH was added. Then 3 ml of a compound-specific 1-butanolol—*n*-heptane mixture were added, the 1-butanol—*n*-heptane ratios being 20:80, 10:90 and 50:50 for propranolol, metoprolol and atenolol, respectively. The tubes were shaken for 15 min and the layers separated by centrifugation. Aliquots of 2.5 ml of the organic layer were transferred to 10-ml conical glass tubes which contained 0.1 ml of 0.1 *N* sulfuric acid. The samples were vortexed for 1 min and centrifuged for 10 min. Finally, aliquots of the aqeous layer (50—75 μl) were injected into the column.

Tissue extraction (Fig. 1)

To each sample of frozen tissue (0.5—1.5 g wet weight) 5 ml of 0.4 *N* per-

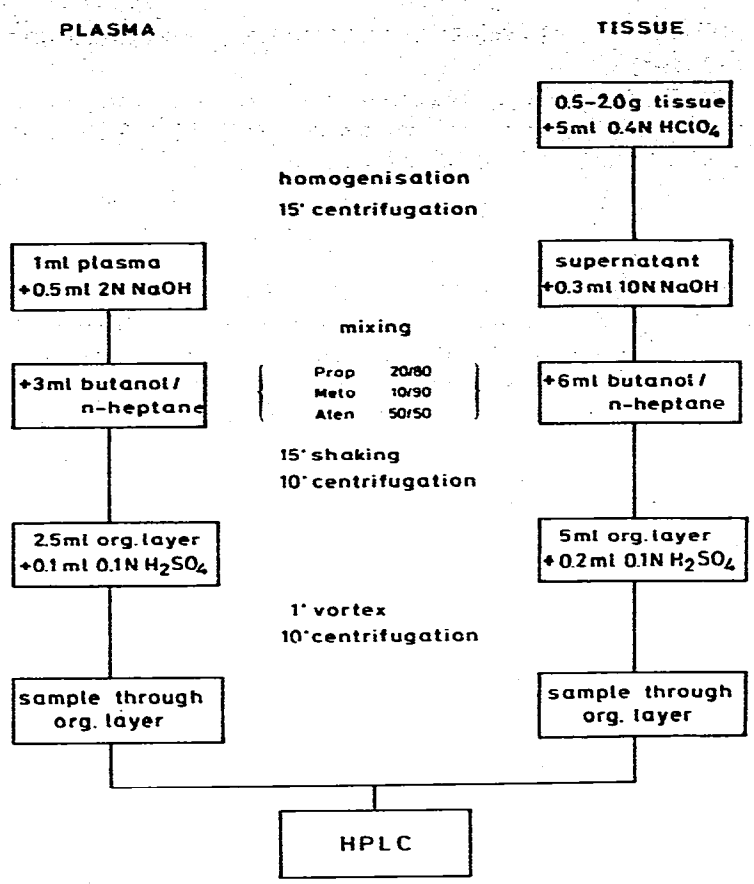


Fig. 1. Schematic outline of the extraction procedure.

chloric acid were added. The samples were homogenized with an Ultra-Turrax homogenizer in stainless-steel tubes at 4°C. After centrifugation at 7000 *g* for 15 min, the supernatants were transferred to 12-ml screw-capped glass tubes containing 0.3 ml of 10 *N* NaOH, and vortexed for 10 sec. Then 6 ml of the 1-butanol—*n*-heptane mixture were added as described above. The two layers were mixed by shaking for 15 min and separated by centrifugation. Aliquots of 5 ml were transferred to 12-ml screw-capped glass tubes and re-extracted with 0.2 ml of 0.1 *N* sulfuric acid as described above. Finally, aliquots of the aqueous layer (10—150 μl) were injected into the column.

Standard curves and recovery studies

Blank plasma and tissue samples from untreated rats were spiked with varying amounts of the compounds. These samples were treated as described above and standard curves of added concentrations versus peak height were calculated. From these data recoveries for each compound were calculated over the whole concentration range.

The range of concentrations for each of the three compounds used to establish standard curves for a specific tissue was chosen according to the tissue

concentrations expected and observed after intravenous injection of equimolar (6 μmol/kg) doses of the drug [30].

In addition, we tested to see if the recovery was dependent on the amount of tissue extracted; in these experiments pieces of lung tissue (250, 500, 750, and 1000 mg wet weight) were spiked with 500 ng of the respective drugs.

RESULTS AND DISCUSSION

As can be seen in Figs. 2 and 3, the extraction of propranolol, metoprolol and atenolol with 1-butanol—*n*-hepatane and the back-extraction into acid yielded clean samples without interfering peaks in the chromatograms. All three compounds were eluted from the column as symmetrical peaks with retention times of 5.5, 3.8 and 4.2 min for propranolol, metoprolol and

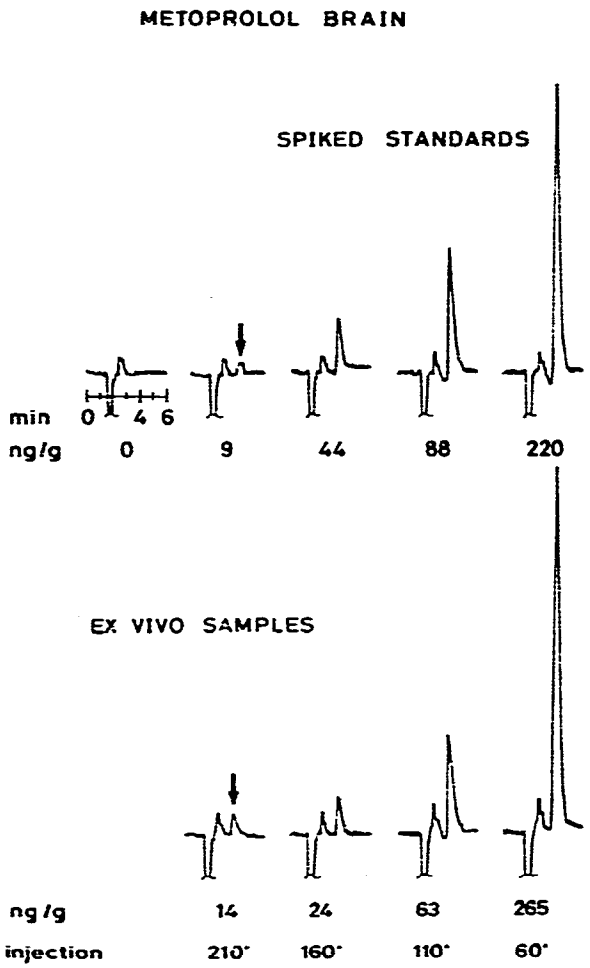


Fig. 2. Representative chromatograms of metoprolol in brain samples. Upper part shows spiked samples in which metoprolol was added at 0—220 ng/g. Lower part shows ex vivo samples in which metoprolol was injected intravenously into rats, and brain tissue was isolated 60—210 min after drug application; the concentrations obtained are indicated.

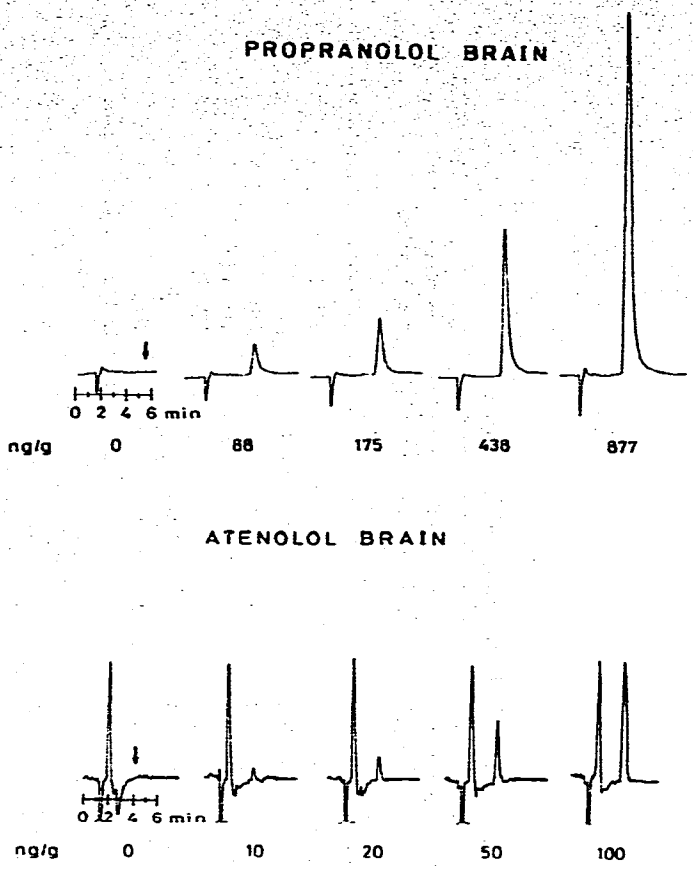


Fig. 3. Representative chromatograms of brain samples spiked with propranolol (upper part) or atenolol (lower part); arrows indicate the respective retention times.

atenolol, respectively. The standard curves showed a linear relationship between compound concentration and peak height.

In Tables II—IV are summarized all the data that were obtained for propranolol, metoprolol and atenolol in plasma, liver, lung, muscle, heart, brain and kidney of the rat. It can be seen that for all compounds in all organs the correlation coefficient between drug concentration and peak height is greater than 0.99. With the system used, the recovery for metoprolol in plasma was 47% and in the tissues it was in the range 56—64% (Table III), while for atenolol recovery was in the range 25—37% (Table IV). The recovery of propranolol greatly varied from 70% for plasma to 16% for liver tissue (Table II). Additional experiments with varying amounts of tissue revealed that the recovery of propranolol is dependent upon the amount of tissue extracted (Fig. 4); thus the limiting factor for this highly lipophilic compound is the ratio of tissue weight to volume of perchloric acid.

The analysis of about 1300 samples within a study on the kinetic behaviour of the three drugs in rat after intravenous administration [30] has demonstrated the applicability of the method. In Fig. 2 chromatograms of ex

TABLE II

DATA FOR PROPRANOLOL OBTAINED FROM SPIKED PLASMA AND TISSUE SAMPLES

Organ	No. of samples	Concentration (ng/sample)	Correlation coefficient (r)	Recovery (% ± S.E.M.)	Tissue weight (g wet weight)	Volume injected (µl)	Detector attenuation
Plasma	11	4.4— 88	0.9977	70.4 ± 2.5	—	75	High
Liver	12	8.8— 438	0.9974	16.1 ± 0.9	1.577 ± 0.036	150	High
Lung	12	88.0—4380	0.9990	38.4 ± 3.2	0.744 ± 0.013	20	Medium
Muscle	11	8.8— 351	0.9976	36.4 ± 3.7	1.188 ± 0.045	100	High
Heart	12	8.8— 351	0.9992	41.6 ± 3.4	0.505 ± 0.008	100	High
Brain	10	88.0—1750	0.9993	25.3 ± 1.3	1.172 ± 0.011	10	Medium

TABLE III

DATA FOR METOPROLOL OBTAINED FROM SPIKED PLASMA AND TISSUE SAMPLES

Organ	No. of samples	Concentration (ng/sample)	Correlation coefficient (r)	Recovery (% ± S.E.M.)	Tissue weight (g wet weight)	Volume injected (µl)	Detector attenuation
Plasma	11	4.4— 26	0.9976	47.2 ± 2.2	—	50	HV 50%
Liver	12	8.8—440	0.9994	58.9 ± 0.8	1.655 ± 0.077	50	High
Lung	11	8.8—440	0.9991	56.4 ± 1.6	0.808 ± 0.015	50	High
Muscle	12	8.8—132	0.9985	58.0 ± 1.5	0.589 ± 0.032	75	HV 50%
Heart	12	8.8—132	0.9986	63.6 ± 1.6	0.486 ± 0.010	75	HV 50%
Brain	12	8.8—440	0.9984	57.5 ± 1.8	1.172 ± 0.014	50	High
Kidney	12	8.8—220	0.9994	64.1 ± 3.3	0.605 ± 0.016	50	High

TABLE IV

DATA FOR ATENOLOL OBTAINED FROM SPIKED PLASMA AND TISSUE SAMPLES

Organ	No. of samples	Concentration (ng/sample)	Correlation coefficient (r)	Recovery (% ± S.E.M.)	Tissue weight (g wet weight)	Volume injected (μl)	Detector attenuation
Plasma	11	20— 500	0.9982	30.6 ± 1.4	—	50	High
Liver	12	100—2000	0.9997	30.6 ± 0.3	1.635 ± 0.078	100	Medium
Lung	12	100—2000	0.9990	37.2 ± 1.3	0.831 ± 0.027	50	Medium
Muscle	12	100—2000	0.9999	37.8 ± 0.4	0.568 ± 0.023	50	Medium
Heart	12	50—1000	0.9999	37.1 ± 1.2	0.522 ± 0.012	75	High
Brain	10	10— 100	0.9940	28.0 ± 0.5	1.197 ± 0.015	100	High
Kidney	12	20— 500	0.9991	25.3 ± 0.6	0.649 ± 0.01	75	High

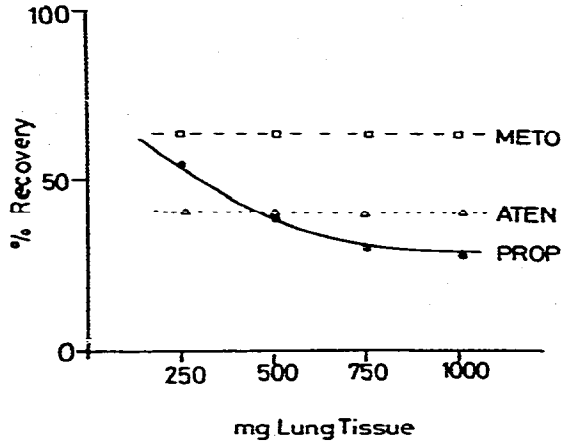


Fig. 4. Recovery of propranolol (PROP), metoprolol (METO) and atenolol (ATEN) in relation to tissue weight.

vivo samples of metoprolol (6 μmol/kg, intravenously) in brain tissue are shown. It can clearly be seen that brain drug concentration declines with time after drug application. Fig. 5 shows the lowest concentrations of propranolol, metoprolol and atenolol measured in this study. It can be seen that also ex vivo all three compounds can be precisely identified and quantified in concentrations as low as 2.2, 2.9 and 5.2 ng/g or ng/ml for propranolol, metoprolol and atenolol, respectively. Also in this study no interfering peaks have been observed (see also Fig. 2). Nevertheless, if a chromatogram were to show inter-

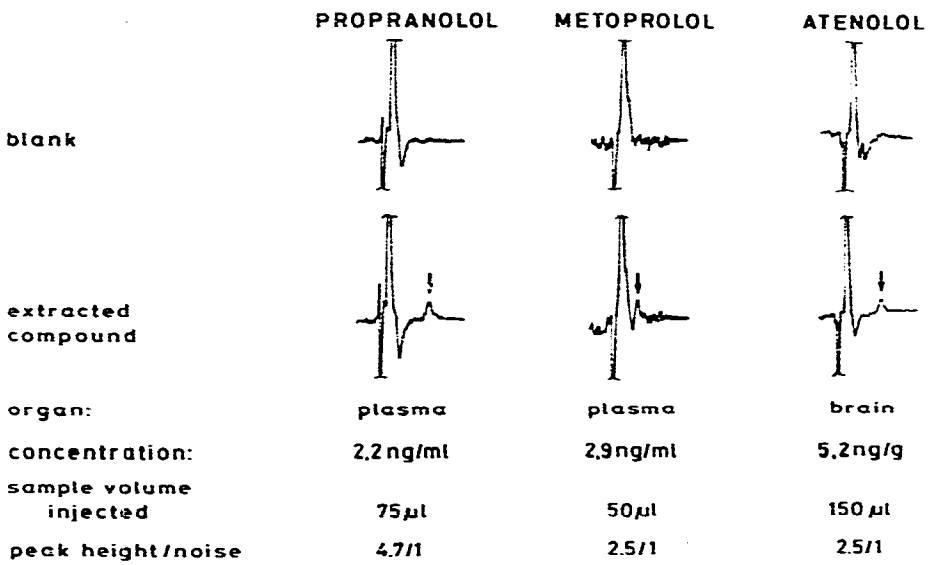


Fig. 5. Chromatograms of the lowest concentrations of propranolol, metoprolol and atenolol measured 3.5 h after intravenous application of 6 μmol/kg.

fering peaks, the retention times of the drugs could be changed by changing the mixture of the mobile phase (ionic pollution) and/or the counter-ion (non-ionic pollution) (Fig. 6). The applicability of this procedure is demonstrated in Fig. 7, which shows a chromatogram from plasma extracted without back-extraction into acid. By changing the counter-ion the propranolol peak could be separated from the interfering peak.

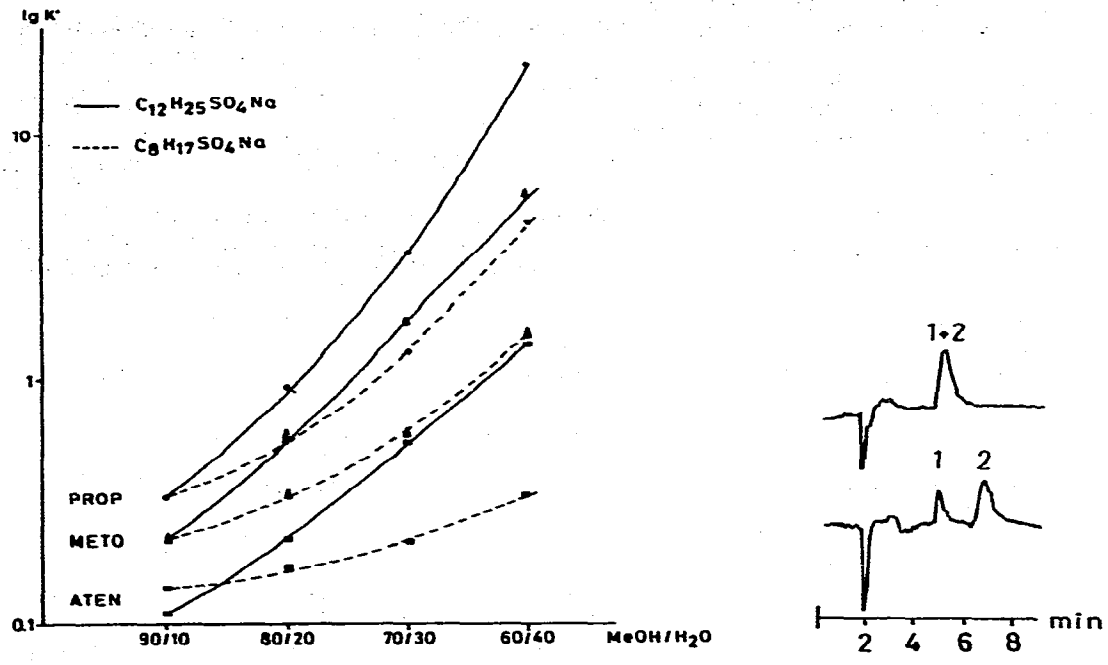


Fig. 6. The capacity factor k' in relation to the mixture of the mobile phase and/or the alkyl chain length of the counter-ion. Abbreviations as in Fig. 4.

Fig. 7. The chromatogram at the top shows an interfering peak (1+2) with the same retention time as propranolol. By changing the counter-ion from $C_8H_{17}SO_4Na$ to $C_9H_{19}SO_4Na$ the propranolol peak (2) could be separated from the unknown interfering peak (1), as can be seen in the bottom chromatogram.

In conclusion, the HPLC method presented here provides for the first time the possibility of investigating the concentration, accumulation and kinetic behaviour of various β-adrenoceptor blocking drugs with great differences in polarity without the necessity of varying greatly the extraction procedure and the chromatographic system. As indicated, this system has been successfully used when studying the acute intravenous kinetic behaviour of propranolol, metoprolol and atenolol in plasma and various tissues of the rat [30] as well as in preliminary studies in man (unpublished data).

If necessary, further methodological variations can be introduced to lower the detection limit for a compound of interest or to detect also drug metabolites as well as other β-blockers of the AOPA type.

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