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DETERMINATION OF (*R*)- AND (*S*)-ALPRENOLOL AND (*R*)- AND (*S*)-METOPROLOL AS THEIR DIASTEREOMERIC DERIVATIVES IN HUMAN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in human plasma. Separation of the enantiomers was accomplished after preparation of diastereomeric derivatives with symmetrical anhydrides of *tert.*-butoxycarbonyl-L-leucine followed by treatment with trifluoroacetic acid at 0°C to remove the *tert.*-butoxycarbonyl group. The separations of the diastereomeric derivatives were performed using a reversed-phase system with μBondapak C_{18} as support and phosphate buffer pH 3.0 with the addition of acetonitrile as the mobile phase. High stability of the chromatographic system was achieved.

The reproducibilities in the determination of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in human plasma were 9.4 and 9.8% (relative standard deviation) for alprenolol and metoprolol, respectively, at drug levels of 0.5 ng/ml.

In two subjects who received single oral doses of alprenolol (100-mg tablet) and metoprolol (50-mg tablet) the plasma levels of the (*R*)-isomers were lower than for the (*S*)-isomers.

INTRODUCTION

Most commercially available adrenergic beta-receptor blocking drugs are racemic mixtures. The (*S*)-forms are considered to cause most of the pharmacological effects [1].

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Recently we developed a high-performance liquid chromatographic (HPLC) technique enabling separation and quantitation of (*R*)- and (*S*)-propranolol as their diastereomeric derivatives with N-trifluoroacetyl-L-proline or L-leucine [2, 3], and we found that the two isomers had different plasma disposition kinetics after giving the racemate to man [2]. Similar results were reported thereafter [4]. Differences in the kinetics of optical isomers may be important clinically and in studies on concentration—effect relationships. It is of general interest to make possible such studies also with other beta-receptor blocking drugs. Therefore we have developed a HPLC method for the separation and quantitation of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in plasma after administration of clinical doses of the racemic drugs to man.

This paper presents studies of the formation rates of the diasteromeric L-leucine derivatives of the two drugs, using the technique described previously [3], as well as the chromatographic behaviour of the diastereomeric derivatives. Plasma concentrations of the (*R*)- and (*S*)-isomers of the two drugs in two healthy volunteers given single doses, 100 mg of alprenolol and 50 mg of metoprolol, are also presented.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was built up from an Altex Model 100 solvent delivery system and a Waters Model U6K injector. A Schoeffel FS 970 L.C. fluorimeter was used as the detector. The detector was operated with an excitation wavelength of 193 nm (for metoprolol determinations) or 198 nm (for alprenolol determinations) without cut-off emission filter. The mass spectrometer was an LKB 2091 equipped with a digital PDP 11/05 computer system and operated in the electron-impact mode at 70 eV. Infrared (IR) spectra were recorded with a Perkin-Elmer 457 spectrophotometer. pH was measured with an Orion Research Model 801 A/ digital pH meter equipped with an Ingold combined electrode Type 401.

Chemicals

Racemic alprenolol hydrochloride, (*R*)- and (*S*)-alprenolol tartrate and racemic metoprolol tartrate were kindly supplied by AB Hässle, Mölndal, Sweden. N-*tert.*-Butoxycarbonyl-L-leucine (Boc-L-Leu) and N-*tert.*-butoxycarbonyl-L-alanine (Boc-L-Ala) were obtained from Sigma (St. Louis, MO, U.S.A.). N,N-Dicyclohexylcarbodiimide and phosgene were obtained from Fluka (Buchs, Switzerland). The support, μBondapak C_{18} (10 μm), was obtained from E. Merck (Darmstadt, G.F.R.). The acetonitrile was of grade S quality and purchased from Rathburn Chemicals (Walkerburn, Great Britain). Triethylamine "zur Synthese" and trifluoroacetic acid (TFA) "für die Spektroskopie" were obtained from E. Merck. The triethylamine was treated overnight with sodium hydroxide pellets to remove water. The sodium hydroxide pellets were removed by filtration and naphthylisocyanate (2%, v/v) was added to the triethylamine before distillation to remove primary and secondary amines [5]. All other chemicals used were of analytical or equivalent grade and were used without further purification.

Chromatographic technique

Chromatographic analyses were performed at room temperature. The mobile phases were prepared from phosphoric acid (final phosphate concentration 0.1 *M*), appropriate amounts of sodium hydroxide (pH was adjusted to 3.0) acetonitrile and water. The mobile phase was degassed in an ultrasonic bath before use. The volume of the mobile phase in the column, V_M, was measured either by injection of water or phosphate buffer pH 7.5.

The asymmetry factor was calculated by drawing a perpendicular to the baseline from the vertex formed by the two peak tangent lines. The back part of the peak baseline divided by the front part gives the asymmetry factor.

Column preparation

The column (100 × 3.2 mm) was made of 316 stainless steel with a polished inner surface, equipped with modified Swagelok connections and Altex stainless-steel frits (2 μm). The column was packed by a modification of the balanced density slurry technique described by Majors [6]. The support was suspended in 10 ml of chloroform and poured into the packing column which was filled by hexane. Acetone was used as driving liquid in the Haskel pump which was operated at 36.2 MPa. After packing the column was washed by pumping 50 ml of acetonitrile through the column followed by 100 ml of acetonitrile—water (50:50). The column was then equilibrated with 150 ml of the mobile phase.

Synthesis of the reagents

Symmetrical anhydrides of Boc-L-Ala and Boc-L-Leu were prepared in two different ways: either by use of phosgene [7] or N,N-dicyclohexylcarbodiimide [8].

Phosgene method. A solution of 1 mmole of the Boc-amino acid and 1.1 mmole of triethylamine in 5.0 ml of methylene chloride was chilled in a dry ice—ethanol bath to −40°C. One half mmole of phosgene (a 20% phosgene solution in toluene) was added and after 5 min the reaction mixture was analysed by IR spectroscopy. The symmetrical anhydrides have three characteristic absorption bands at 1830, 1750 and 1720 cm^{-1} [7]. The reagents were stored at −20°C until they were used.

N,N-Dicyclohexylcarbodiimide method. To a solution of 1 mmole of the Boc-amino acid in 3.0 ml of methylene chloride was added 0.5 mmole of N,N-dicyclohexylcarbodiimide (2.0 ml of a 0.25 *M* N,N-dicyclohexylcarbodiimide solution in methylene chloride) and allowed to react for 1 h at 0°C. The reaction mixture was filtered to remove the urea formed during the reaction and finally analyzed by IR spectroscopy as above.

Evaluation of reaction conditions

A reaction scheme for the derivatization process is given in Fig. 1.

Reaction of alprenolol and metoprolol with the leucine reagent. (1) Racemic alprenolol hydrochloride and metoprolol tartrate corresponding to 97 and 104 ng as base, respectively, were added to 1.0 ml of 1 *M* carbonate buffer pH 9.9. Six millilitres of water-saturated diethyl ether were added and the tubes were agitated for 15 min. After centrifugation at 4200 *g* for 5 min, 5.0 ml of the

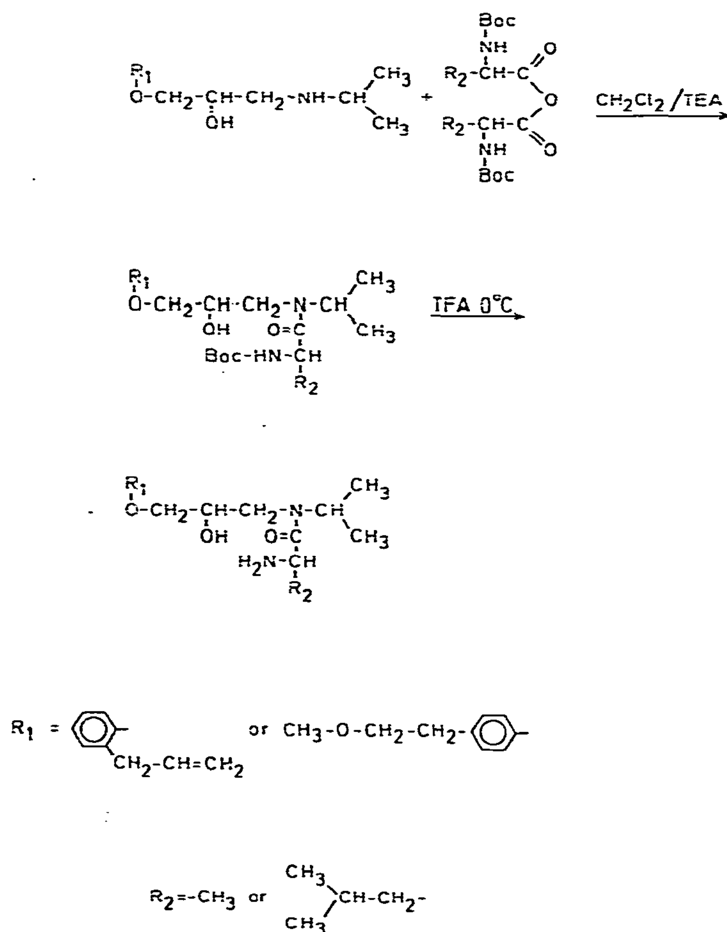


Fig. 1. Reaction of alprenolol and metoprolol with symmetrical anhydrides of Boc-amino acids. TEA = triethylamine.

ether phases were evaporated at 35°C in a stream of dry nitrogen.

(2) The residues were dissolved in 250 μl of a solution of triethylamine in methylene chloride (for alprenolol 89 μmoles of triethylamine and for metoprolol 35.6 μmoles of triethylamine, see below) and 100 μl of the reagent (Boc-L-Leu) were added. After suitable intervals samples were taken out in duplicate and evaporated under nitrogen at 35°C. Two millilitres of 0.1 *M* sodium hydroxide were added to the tubes and the tubes were agitated for 10 min to hydrolyse excess reagent. Six millilitres of ether were added and the solutions were extracted with ether for 15 min. After centrifugation 5.0 ml of the ether phases were evaporated with dry nitrogen at 35°C.

(3) The residues were treated for 10 min with 250 μl of TFA in an ice-bath (see below). The reaction was stopped by addition of 2.0 ml of 2 *M* sodium hydroxide. The solutions were extracted with 6.0 ml of ether and 5.0 ml of the ether phases were collected and extracted with 100 μl (for metoprolol) or 150 μl (for alprenolol) of 0.1 *M* phosphoric acid.

(4) An aliquot of 94 μl (for metoprolol) or 145 μl (for alprenolol) of the phosphoric acid extracts was injected on to the column. The alprenolol-L-Leu derivatives were separated using a mobile phase consisting of 35% acetonitrile in phosphate buffer pH 3.0. The separation of the metoprolol-L-Leu derivatives was performed using 30% acetonitrile in phosphate buffer pH 3.0.

Dependence of the yield on the triethylamine concentration. The dependence of the yield of the alprenolol-Boc-L-Leu and the metoprolol-Boc-L-Leu derivatives on the triethylamine concentration were evaluated by reaction of alprenolol and metoprolol according to the procedure given above. The amount of triethylamine in the reaction mixture was varied between 1.78 and 124.6 μmoles. The samples were allowed to react for 30 min with the Leu reagent.

Removal of the Boc group from the alprenolol-Boc-L-Leu and metoprolol-Boc-L-Leu derivatives. One millilitre of a solution containing 6.3 μg of racemic metoprolol tartrate per ml or 4.5 μg of racemic alprenolol hydrochloride per ml was extracted and treated as described in (1) and (2) above with the exception that 20 ml of ether were used for the extraction of the derivatives (2).

One-millilitre portions of the ether phase were taken out and evaporated in a stream of dry nitrogen at 35°C. The tubes were cooled in an ice-bath and 250 μl of TFA were added. After suitable intervals the reaction was stopped in duplicate tubes and treated as in (3) above. Aliquots of the phosphoric acid extracts were injected on to the column. The mobile phases described in (4) above were used.

Standard curves and analytical procedure for quantitation of (R)- and (S)-metoprolol and (R)- and (S)-alprenolol in human plasma

Standard curves were prepared by adding known amounts of racemic alprenolol hydrochloride (corresponding to 1—97 ng as base) and metoprolol tartrate (corresponding to 1—104 ng as base) to 1.0 ml of 1 *M* carbonate buffer pH 9.9 and 1.0 ml of drug-free plasma (for standard samples). Standard and original plasma samples (1.0 ml) were treated according to the procedure given under *Reaction of alprenolol and metoprolol with the leucine reagent.* Samples containing alprenolol were reacted for 30 min, while a 15-min reaction time was used for the samples containing metoprolol.

Standard curves were constructed by plotting the peak height versus the sample concentration.

Identification of the derivatives

Racemic metoprolol tartrate and alprenolol hydrochloride corresponding to about 50 μg as base of the two substances were extracted and derivatised as described above. The pH of the phosphoric acid extracts (3) was adjusted to 11 with sodium hydroxide and the derivatives were extracted to ether. An aliquot of each ether phase was collected and evaporated in a stream of dry nitrogen at 35°C. The residues were dissolved in 50 μl of methylene chloride and an aliquot was injected directly into the ion source of the mass spectrometer which was operated with an electron energy of 70 eV.

RESULTS AND DISCUSSION

Extraction procedures

Alprenolol and metoprolol are secondary amines with a pK_a of 9.63 ± 0.02 and 9.68 ± 0.02, respectively [9]. Alprenolol is quantitatively extracted by ether from 1 *M* carbonate buffer pH 9.9 with a phase-volume ratio (V_{org}/V_{aq}) of 3.0. Under the same conditions 98.5% of metoprolol is extracted by ether. These extraction studies were performed with alprenolol hydrochloride and metoprolol tartrate concentrations of 96.1 and 139.7 μg/ml, respectively.

After the removal of the Boc protection group, the L-Leu derivatives of alprenolol and metoprolol were extracted by ether from the 2 *M* sodium hydroxide solution. The degree of extraction of these derivatives was determined by quantitation of the diastereomeric derivatives in the two phases using the liquid chromatographic systems described under Experimental (4). It was found that the two diastereomeric derivatives of alprenolol were quantitatively extracted to the organic phase, while 94.7% of the L-Leu derivative of (*S*)-metoprolol and 98.7% of the (*R*)-metoprolol derivative was extracted into ether under the same conditions. This study was performed using alprenolol- and metoprolol-L-Leu concentrations of 4.9 and 5.0 μg/ml, respectively.

The L-Leu derivatives of alprenolol and metoprolol were extracted by 0.1 *M* phosphoric acid in the last extraction step. The degree of extraction of the derivatives by the phosphoric acid was determined by quantitation of the derivatives in the two phases chromatographically. With a phase-volume ratio (V_{org}/V_{aq}) of 50, the two metoprolol derivatives were found to be quantitatively extracted into the aqueous phase. The degree of extraction of the more lipophilic alprenolol derivatives was 91.7 and 85.6% for the (*S*)- and (*R*)-alprenolol derivatives, respectively, with a phase-volume ratio (V_{org}/V_{aq}) of 33.3. The same sample concentrations as for the above extraction study were used. All extraction yields are the mean values of two determinations.

Synthesis of the reagent

The symmetrical anhydride of Boc-leucine was prepared in two different ways, either by reaction of the triethylammonium salt of the Boc-amino acid with phosgene or by reaction of Boc-leucine with N,N-dicyclohexylcarbodiimide. It was, however, sometimes observed that by-products are formed when the phosgene method is used. Fig. 2A demonstrates an IR spectrum of a contaminated symmetrical anhydride of Boc-leucine. The peaks at 1685 and 1785 cm^{-1} are probably the result of an excess of phosgene in the reaction mixture [7].

The second method, in which N,N-dicyclohexylcarbodiimide was used for the synthesis of the Leu reagent, is easier to reproduce and the peaks at 1685 and 1785 cm^{-1} in the IR spectrum have never been observed using this method. An IR spectrum of a symmetrical anhydride of leucine of good quality is demonstrated in Fig. 2B.

Preparation of the Leu reagent by the latter method has the disadvantage that it is slower than the phosgene method.

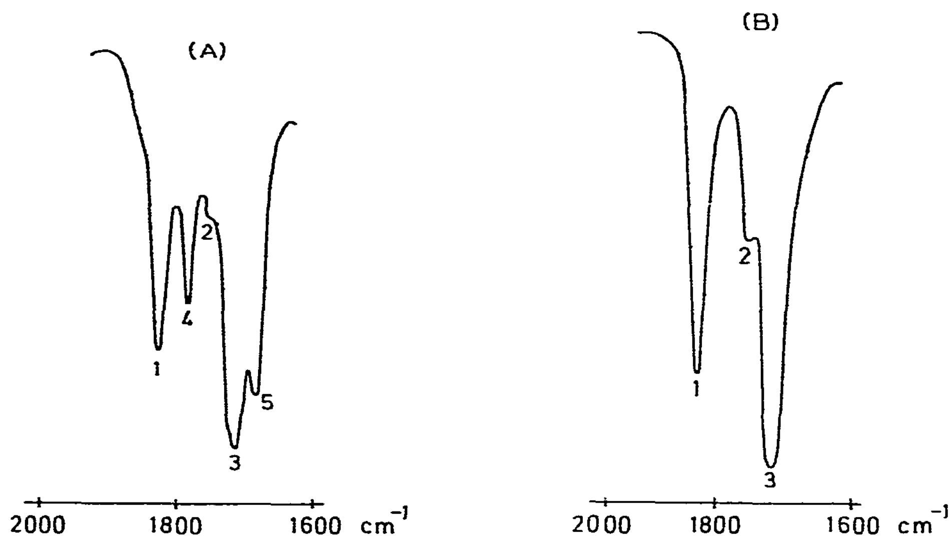


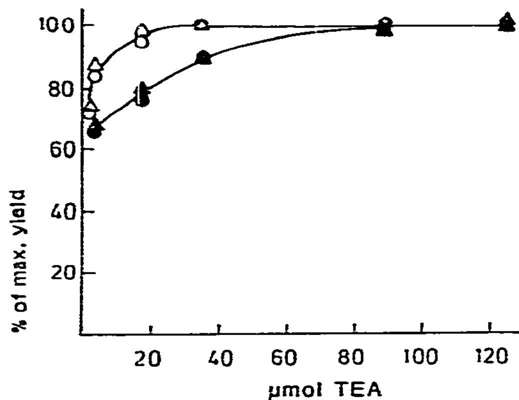
Fig. 2. IR spectra of symmetrical anhydrides of Boc-L-leucine. (A) Contaminated reagent prepared by the phosgene method. (B) Reagent of good quality prepared by the N,N-dicyclohexylcarbodiimide method. Peaks 1 (1830 cm^{-1}) and 2 (1760 cm^{-1}) arise from the carbonyl group of the symmetrical anhydride; peak 3 (1720 cm^{-1}) arises from the carbonyl group of the Boc protection group. Peaks 4 (1790 cm^{-1}) and 5 (1690 cm^{-1}) are by-products [7].

Derivatization procedure

Diastereomeric derivatives of alprenolol and metoprolol were prepared by reaction with a symmetrical anhydride of leucine, and by use of triethylamine as the catalyzing agent. This derivatization technique has been described in a previous paper for preparation of diastereomeric derivatives of propranolol [3].

It was observed that the content of triethylamine in the reaction mixture influences the yield of the diastereomeric derivatives of alprenolol and metoprolol. This study was performed by reacting the two substances with the Leu reagent for 30 min and by varying the triethylamine content in the reaction mixture between 1.78 and 124.6 μmoles. The result of this study is demonstrated in Fig. 3, and it was observed that the maximum catalyzing effect was obtained with 36 and 90 μmoles of triethylamine, corresponding to a molar excess of triethylamine of 95,000 and 222,000 times for metoprolol and alprenolol, respectively.

The relationship between the yield of the Boc-L-Leu derivatives of alprenolol and metoprolol and the reaction time was evaluated as described under Experimental. Fig. 4 demonstrates the result of this study; maximum yield of the metoprolol derivatives was obtained after 15 min whereas a 30-min reaction time was required for alprenolol. No significant level of underivatized substance was detected in the reaction mixture after the reaction with the Leu reagent for the stated times. This was assessed by reaction of alprenolol and metoprolol according to the procedure given under Experimental. After the reaction with the Leu reagent and extraction with ether (paragraph 2 of the procedure), an aliquot of the ether phase was extracted with 0.1 *M* phosphoric acid, which was analyzed chromatographically.



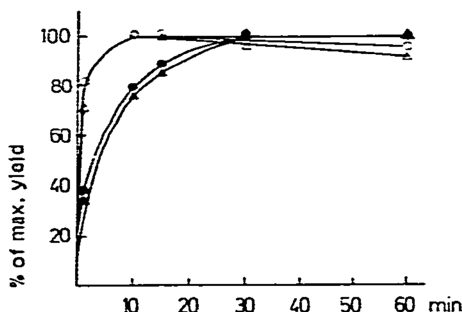


Fig. 3. Influence of triethylamine concentration on the yield of alprenolol- and metoprolol-Boc-L-Leu. (o), (*S*)-Metoprolol-Boc-L-Leu; (△), (*R*)-metoprolol-Boc-L-Leu; (•), (*S*)-alprenolol-Boc-L-Leu; (▲), (*R*)-alprenolol-Boc-L-Leu.

Fig. 4. Formation rates of the alprenolol- and metoprolol-Boc-L-Leu derivatives. Symbols as in Fig. 3.

Deprotection of the Boc-L-Leu derivatives of alprenolol and metoprolol

The last step in the derivatization procedure is deprotection of the primary amino group of the Boc-L-Leu derivatives of alprenolol and metoprolol. The Boc group is removed by reaction with TFA in an ice-bath at 0°C as described previously [3]. Maximum yield of the L-Leu derivatives of the two substances is obtained after 10 min, and no indications of non-completion of this reaction were found. In accordance with previous findings for propranolol, the Boc-L-Leu derivatives of the (*R*)-isomers of alprenolol and metoprolol are more slowly hydrolyzed than the corresponding (*S*)-isomer derivatives [3]. This effect is demonstrated in Fig. 5 where the peak area ratios (*S*/*R*) for the diastereomeric pairs are plotted against the reaction time. From Fig. 5 it can be seen that

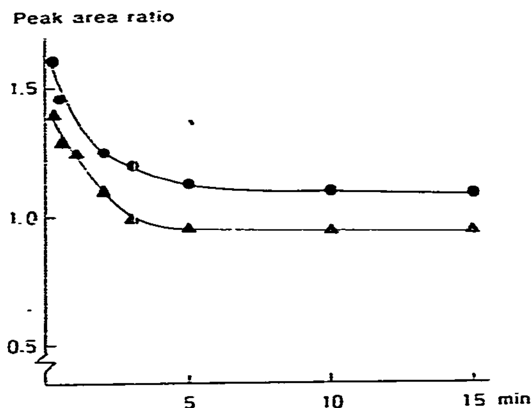


Fig. 5. *S*/*R* peak area ratios after different times of hydrolysis by TFA. (•), Alprenolol-L-Leu; (▲), metoprolol-L-Leu.

initially the S/R peak area ratios rapidly decrease, but after 5 min the curves level off and reach constant values at about 1.1 and 0.94 for the alprenolol and the metoprolol derivatives, respectively. The discrepancy from 1.0 of the S/R peak area ratios is probably caused by the extraction procedure used in the isolation step. It was shown under *Extraction procedures* that the two diastereomeric alprenolol derivatives are not extracted to the same extent in the last extraction step (into 0.1 *M* phosphoric acid) and a peak area ratio (S/R) of 1.07 is created by this extraction. It was also observed that the metoprolol-L-Leu derivatives are not extracted to the same degree into ether from a 2 *M* sodium hydroxide solution (see *Extraction procedures*). This extraction step creates a ratio (S/R) of 0.96 which is in good agreement with the 0.94 found in Fig. 5.

Racemization test

To confirm that the discrepancies from 1.0 of the S/R peak area ratios are not caused by racemization during the derivatization procedure, (S)- and (R)-alprenolol were reacted separately according to the procedure given under Experimental. Fig. 6A and B show the result of this study. Only one peak in each chromatogram was detected, corresponding to the L-Leu derivatives of (S)- and (R)-alprenolol, respectively. This is in accordance with previous findings for propranolol derivatized with symmetrical anhydrides of Boc-amino acids [3].

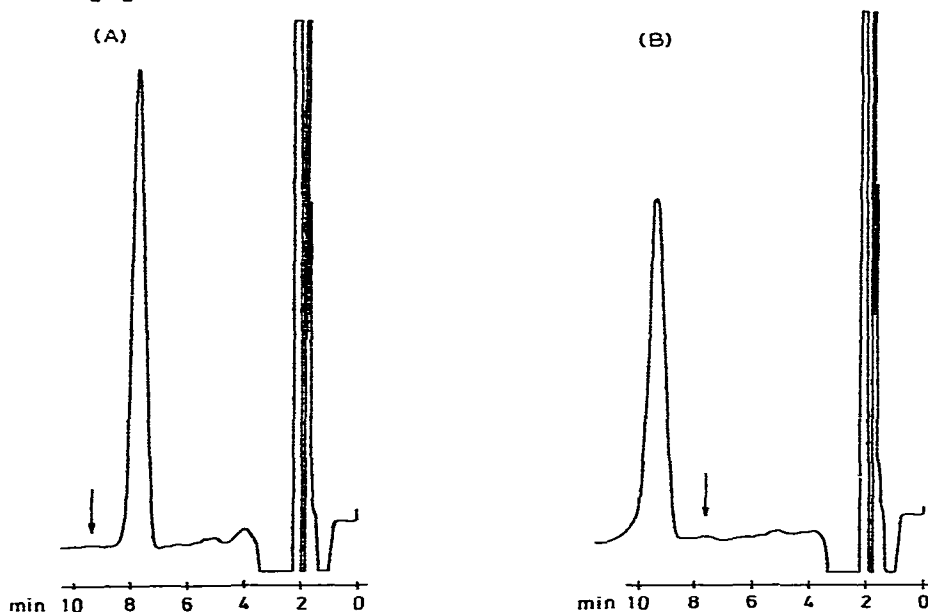


Fig. 6. Racemization test. (A) (S)-Alprenolol (48.5 ng as base) derivatized according to the procedure given under Experimental. Arrow indicates the retention time for the (R)-alprenolol-L-Leu derivative. (B) (R)-Alprenolol (48.1 ng as base) derivatized as above. Arrow indicates the retention time for the (S)-alprenolol-L-Leu derivative. Column: 100 × 3.2 mm packed with μBondapak C_{18} (10 μm). Mobile phase: 35% acetonitrile in phosphate buffer (pH 3.0); flow-rate, 0.5 ml/min.

Identification of the derivatives

The identity of the derivatives was confirmed by mass spectrometry. The fragmentation pattern coincided with that for the L-Leu derivatives of propranolol and the base peaks were in accordance with the propranolol-L-Leu derivatives m/e = 86 [3]. Molecular ions with low intensity were also present in the spectra at m/e 362 and 380 for the alprenolol and the metoprolol derivatives, respectively. Peaks at m/e = 229 were also obtained for the two derivatives, which arise from cleavage of the ether linkage.

Chromatographic studies

The diastereomeric derivatives of alprenolol and metoprolol were separated using µBondapak C_{18} as support and acetonitrile in phosphate buffer pH 3.0 as the mobile phase. In a previous study the L-Leu derivatives of propranolol were separated using LiChrosorb RP-18 as support and a mobile phase consisting of phosphate buffer (pH 3.0)—acetonitrile, with addition of the tertiary amine N,N-dimethyloctylamine [3]. Addition of the tertiary amine to the mobile phase was a prerequisite for obtaining good chromatographic performance of the propranolol derivatives. It was observed, however, that by use of µBondapak C_{18} as support the tertiary amine could be excluded from the mobile phase without loss of column efficiency or peak symmetry, which also has been observed previously during chromatography of secondary and tertiary amines [10].

The capacity factor of the L-Leu derivatives of alprenolol and metoprolol can be regulated over a wide range by varying the content of acetonitrile in the mobile phase; this is demonstrated in Fig. 7. The selectivity increases with de-

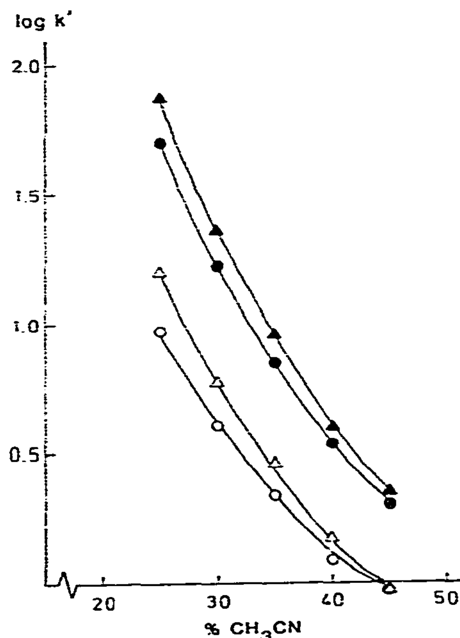


Fig. 7. Regulation of the capacity factor (k') by the acetonitrile concentration. Column and flow-rate: as in Fig. 6. Mobile phase: phosphate buffer (phosphate concentration 0.1 M) pH 3.0 with different concentrations of acetonitrile. Symbols as in Fig. 3.

creasing concentration of acetonitrile, and the separation factor, α, ranges between 1.0–1.67 and 1.10–1.49 for the metoprolol and the alprenolol derivatives, respectively. Baseline separation of the diastereomeric alprenolol and metoprolol derivatives was obtained using mobile phases containing 35 and 30% (v/v) acetonitrile in phosphate buffer (pH 3.0), respectively. Typical chromatographic data for the derivatives using these mobile phases are given in Table I. The separation efficiency shows a strong dependence of the capacity factor and a drastic increase of H (the height of a theoretical plate) was observed with capacity factors below 5. The alanine derivatives of the two drugs were also prepared and tested but the separation factors obtained were lower compared to the L-Leu derivatives. It was also observed that no resolution at all was obtained by chromatography of the Boc-L-Leu derivatives of alprenolol and metoprolol. This is in accordance with a previous observation during chromatography of the Boc-L-Leu derivatives of 3,4-dihydroxyphenylalanine [11]. The high selectivity obtained between the two diastereomeric pairs by the coupling of L-Leu to the two substances contributes to the high sensitivity obtained with the described method. Large separation factors allow the use of a short column, which gives low interstitial volumes and an increase in the maximum concentration in the eluted peak [12].

TABLE I

CHROMATOGRAPHIC DATA

Support: μBondapak C_{18} (10 μm) in a 100 × 3.2 mm column. Mobile phase: phosphate buffer (pH 3.0) containing 30% (for metoprolol derivatives) or 35% (v/v) acetonitrile (for alprenolol derivatives); flow-rate, 0.5 ml/min.

Compound	k'	α	H (μm)	Asymmetry factor
(*S*)-Alprenolol-L-Leu	7.16	1.27	66	1.07
(*R*)-Alprenolol-L-Leu	9.11		78	0.84
(*S*)-Metoprolol-L-Leu	4.03	1.45	87	1.11
(*R*)-Metoprolol-L-Leu	5.86		120	0.96

Fluorimetric detection

Fluorimetric detection was used for quantitation of the diastereomeric derivatives of alprenolol and metoprolol in human plasma. This detection system gave excellent sensitivity and made possible the selective detection of the derivatives of the drugs in plasma. Fig. 8 demonstrates the relative fluorimetric response obtained with different excitation wavelengths. The alprenolol derivatives gave maximal response with an excitation wavelength of 198 nm, while 193 nm was the maximum for the metoprolol derivatives. No interference from endogenous compounds in human plasma was observed in the chromatograms using the excitation wavelengths above.

Determination of (R)- and (S)-alprenolol and metoprolol in human plasma

The plasma concentrations of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol were determined using the method described under Experimental.

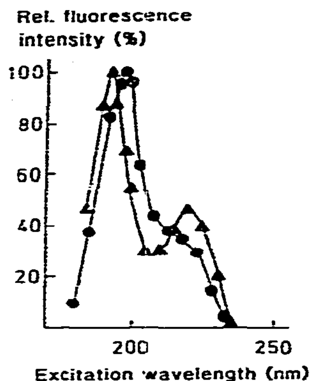


Fig. 8. Relative fluorimetric response at different excitation wavelengths. (●), Alprenolol-L-Leu; (▲), metoprolol-L-Leu.

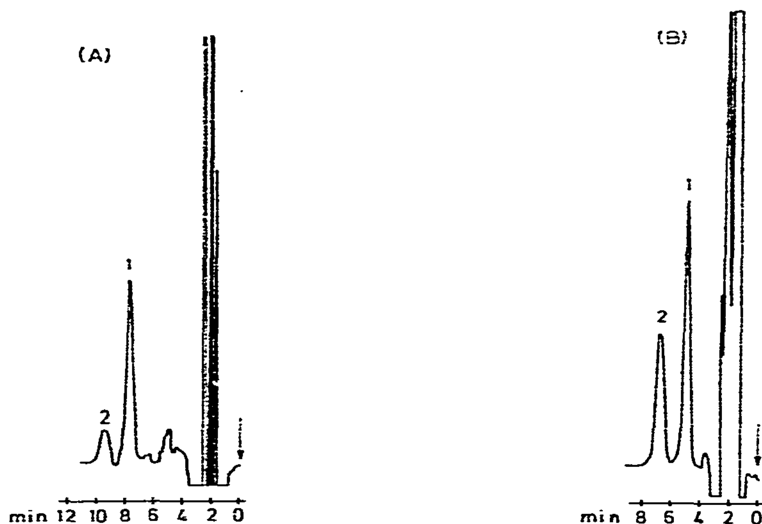


Fig. 9. Separation of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol extracted from human plasma. (A) 1 = (*S*)-Alprenolol-L-Leu (6.6 ng/ml), 2 = (*R*)-alprenolol-L-Leu (1.6 ng/ml). Conditions as in Fig. 6. (B) 1 = (*S*)-Metoprolol-L-Leu (22.5 ng/ml), 2 = (*R*)-metoprolol-L-Leu (14.2 ng/ml). Mobile phase: 30% acetonitrile in phosphate buffer pH 3.0. Column and flow-rate as in Fig. 6.

Fig. 9A and B demonstrate the separation of the (*R*)- and (*S*)-isomers of the two drugs as their diastereomeric derivatives. The plasma samples were obtained from healthy male volunteers 1.12 and 1.25 h after oral administration of a 100-mg Aptin tablet and a 50-mg Seloken tablet, respectively. Standard curves used for quantitation of the isomers in plasma were constructed by plotting the peak height against the sample concentrations. The standard curves were linear in the studied concentration ranges 0.5—48.5 and 0.5—52 ng/ml for alprenolol and metoprolol, respectively; the curves are shown in Fig. 10. Correlation coefficients were in all cases better than 0.9992.

Standard samples were extracted after addition of drug-free plasma to avoid differences in the recoveries of the drugs from original plasma and standard samples due to protein binding.

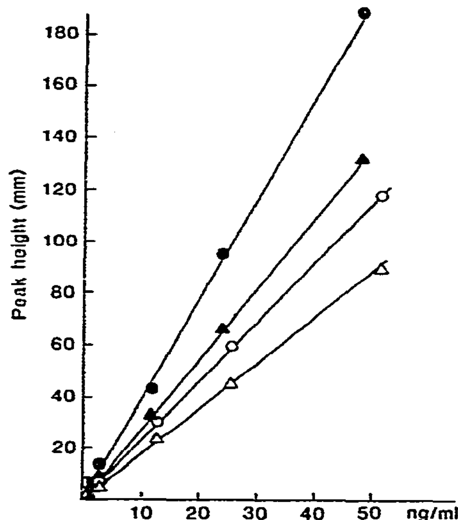


Fig. 10. Standard curves for alprenolol and metoprolol. (•), (*S*)-alprenolol-L-Leu; (▲), (*R*)-alprenolol-L-Leu: (○), (*S*)-metoprolol-L-Leu; (△), (*R*)-metoprolol-L-Leu.

The reproducibility of the method was studied at three different concentrations and the results are summarized in Table II.

The minimum detectable concentrations were 0.2 and 0.4 ng/ml for alprenolol and metoprolol, respectively. The signal-to-noise ratio was 3:1 for each compound at these concentrations.

The column used for this study has a high stability with the mobile phases used. No significant change in the separation efficiency has been observed during continuous use of the column for more than 5 months.

TABLE II

REPRODUCIBILITY OF (*R*)- AND (*S*)-ALPRENOLOL AND (*R*)- AND (*S*)-METOPROLOL DETERMINATIONS IN HUMAN PLASMA

The relative standard deviation (S.D.) calculated for $n = 6$.

Alprenolol		Metoprolol	
Sample conc. (ng/ml)	Relative S.D. (%)	Sample conc. (ng/ml)	Relative S.D. (%)
0.5	9.4	0.5	9.8
12.1	6.7	12.5	6.5
48.5	4.9	52.0	5.1

Pharmacokinetics

To test the applicability of the methods for studies in patients alprenolol and metoprolol were given to two subjects.

The plasma concentrations of (*R*)- and (*S*)-alprenolol in one subject (A) who received a single oral 100-mg dose of racemic alprenolol is shown in Fig. 11. The concentration of (*S*)-alprenolol was much higher than that of (*R*)-alprenolol This subject (subject 7 in ref. 13) had earlier received alprenolol and, as in this

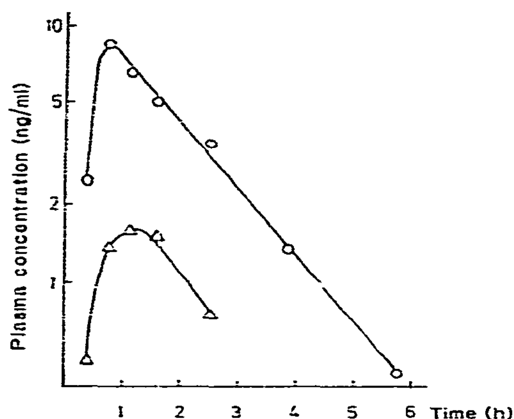


Fig. 11. Plasma concentrations of (*R*)-alprenolol (△) and (*S*)-alprenolol (○) in one subject (A) receiving a 100-mg commercial tablet.

study, had low plasma concentrations of the drug. The same subject has also been shown to achieve unusually high plasma concentrations of the active metabolite 4-hydroxyalprenolol. It is likely that most of accumulated 4-hydroxyalprenolol is the (*R*)-form, which should be inactive. Thus studies estimating relative activities of alprenolol and its active metabolites [14] should reach higher precision if one also considers the separate kinetics of the (*R*)- and the (*S*)-isomers. The above subject also had lower availability of (*R*)- than of (*S*)-propranolol after a single oral dose (40 mg) of racemic propranolol (subject B, Fig. 3 in ref. 2).

In the present study this subject also received a single oral dose (50-mg tablet) of racemic metoprolol. As shown in Fig. 12, subject A also had a lower plasma concentration of (*R*)-metoprolol and thus has lower plasma concentrations of the (*R*)-isomers of three different beta-receptor blocking drugs, probably indicating that his liver oxidizes the (*R*)-isomers of these drugs faster than the (*S*)-isomers. Fig. 12 also shows that another subject (B) had similar con-

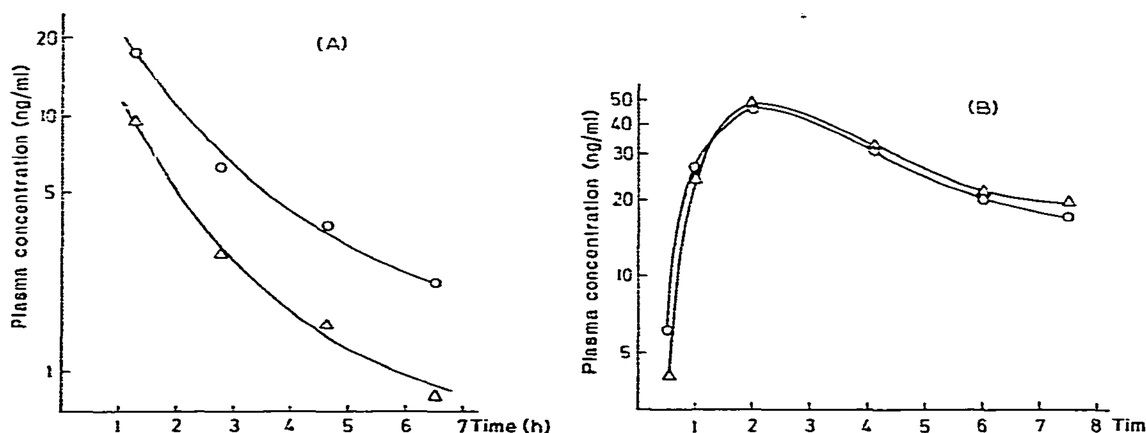


Fig. 12. Plasma concentrations of (*R*)-metoprolol (△) and (*S*)-metoprolol (○) in two subjects (A and B) receiving 50-mg commercial tablets.

centrations of (*R*)- and (*S*)-metoprolol after receiving racemic metoprolol. There seems to be an individual variation in the concentration ratios of the (*R*)- and the (*S*)-isomers. We have recently observed this for propranolol [15].

In conclusion, our HPLC method has enabled simultaneous determination of the (*R*)- and (*S*)-isomers of alprenolol and of metoprolol after giving clinial doses of commercially available tablets. The results show that the isomers can have different kinetics. Therefore it is worthwhile to investigate if this is important clinically.

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REFERENCES

1 A.M. Barret and V.A. Cullum, Brit. J. Pharmacol., 14 (1968) 43.
2 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109.
3 J. Hermansson, Acta Pharm. Suecica, 19 (1982) in press.
4 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643.
5 L.J. Durham, D.J. McLeod and J. Cason, Org. Syn., 4 (1963) 555.
6 R.R. Majors, Anal. Chem., 44 (1972) 1722.
7 T. Wieland, F. Flor and C. Birr, Justus Liebigs Ann. Chem., (1973) 1595.
8 F.M.F. Chen, K. Kuroda and N.L. Benoiton, Synthesis, 12 (1978) 1928.
9 AB Hässle, Mölndal, personal communication.
10 A. Sokolowski and K.-G. Wahlund, J. Chromatogr., 189 (1980) 299.
11 J. Hermansson and B. Wiese, Chromatographia, 14 (1981) 529.
12 G. Schill, Separation Methods for Drugs and Related Organic Compounds, Apotekarsocieteten, Stockholm, 1978, p. 69.
13 G. Alvan, M. Lind, B. Mellström and C. von Bahr, J. Pharmacokinet. Biopharm., 5 (1977) 193.
14 P. Collste, K.-O. Borg, H. Aström and C. von Bahr, Clin. Pharmacol. Ther., 25 (1979) 416.
15 C. von Bahr, J. Hermansson and M. Lind, in preparation.