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Short Communication

Reversed-phase high-performance liquid chromatographic analysis of atenolol enantiomers in plasma after chiral derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate

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ABSTRACT

A sensitive high-performance liquid chromatographic method for the determination of the enantiomers of atenolol in rat plasma has been developed. Racemic atenolol and practolol (internal standard) were extracted from alkalinized plasma (pH 12) into dichloromethane containing 3% (v/v) heptafluoro-1-butanol, and the organic layer was evaporated. The samples were derivatized with (+)-1-(9-fluorenyl)ethyl chloroformate at pH 8.5 for 30 min. After removal of excess reagent, the diastereomers were extracted into dichloromethane. The diastereomers were separated on a Microspher C_{18} column (3 μm) with a mobile phase of acetonitrile–sodium acetate buffer (0.01 *M*, pH 7) (50:50, v/v) at a flow-rate of 0.8 ml/min. Fluorescence detection (λ_{ex} = 227 nm, λ_{em} = 310 nm) was used. When 100 μl of plasma were used, the quantitation limit was 10 ng/ml for the atenolol enantiomers. The assay was applied to measure concentrations of atenolol enantiomers in plasma after intravenous administration of racemic atenolol to rats.

INTRODUCTION

Atenolol, 2-[*p*-(2-hydroxy-3-(isopropylamino)propoxy)phenyl]acetamide (Fig. 1), is a cardioselective β-adrenoceptor blocking agent marketed as a racemic mixture. Methods presently available to quantify atenolol enantiomers simultaneously in biological fluids include reversed-phase high-performance liquid chromatography (RP-HPLC) after chiral derivatization [1–3] and HPLC on a chiral α_1-AGP column after achiral derivatization [4].

A highly fluorescent reagent, (+)-1-(9-fluorenyl)ethyl chloroformate (Flec), was introduced as a chiral derivatizing reagent for amino acid enantiomers by Einarsson *et al.* [5]. We have developed a highly sensitive method for the enantioselective chromatographic analysis of *R*-(+)- and *S*-(−)-atenolol in rat plasma using Flec as the chiral derivatizing reagent.

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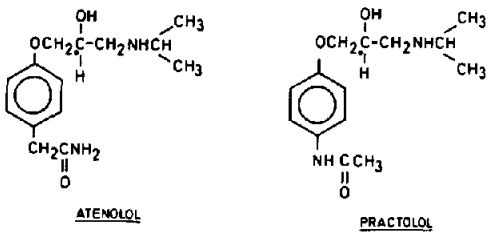

Fig. 1. Structures of atenolol and practolol. The asterisks indicate the asymmetric carbon atoms.

EXPERIMENTAL

Chemicals

Racemic atenolol (base) was purchased from Sigma (St. Louis, MO, USA). *S*-(−)-Atenolol was kindly provided by Nobel Chemicals (Karlskoga, Sweden). Racemic practolol (Fig. 1) was obtained from ICI (Macclesfield, UK). 2,2,3,3,4,4,4-Heptafluoro-1-butanol (96%) and *cis*-1-hydroxy-D-proline (99%) were obtained from Aldrich (Brussels, Belgium). Flec (Eka Nobel, Sweden) was from Biorad (Eke, Belgium). Dichloromethane was LiChrosolv grade from E. Merck (Darmstadt, Germany) and acetonitrile (Far UV), HPLC grade, was purchased from Lab Scan (Almere, Netherlands). All other chemicals and reagents used were of analytical grade from E. Merck.

Instrumentation and chromatography

RP-HPLC was performed using a Varian Star 9010 solvent-delivery system (Sunnyvale, CA, USA), a 7125 Rheodyne manual injector (Cotati, CA, USA) with a 10-μl loop, and a Microspher C_{18} column, 100 mm × 4.6 mm I.D., 3 μm (Chrompack, Antwerp, Belgium). The mobile phase was acetonitrile–sodium acetate buffer (0.01 *M*, pH 7) (50:50, v/v), pumped at a flow-rate of 0.8 ml/min at 25°C. The column eluent was monitored on a Hewlett Packard 1046 A programmable fluorescence detector (Avondale, PA, USA) set at an excitation wavelength of 227 nm and an emission wavelength of 310 nm. The fluorescence output was recorded on a Hewlett Packard 3390 A integrator.

Standard solutions

Stock solutions of atenolol and the internal standard, practolol, were prepared by dissolving the racemic salts in methanol to a final concentration of 1 mg/ml (free base) and were stored at −20°C. Working solutions were obtained by further dilution of the stock solutions with methanol.

Extraction procedure

To 100 μl (or 25 or 50 μl for the samples in which high atenolol concentrations are expected) of plasma in a glass-stoppered tube, 5 μl of the internal standard solution (500 ng), 50 μl of glycine buffer (0.5 *M*, pH 12), 50 μl of 2 *M* sodium

hydroxide and 1 ml of saturated sodium chloride solution were added. The mixture was extracted with 4 ml of dichloromethane containing 3% (v/v) heptafluoro-1-butanol for 10 min and centrifuged (3015 *g*) for 5 min (4°C). The organic phase was transferred to a conical glass centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at room temperature.

Derivatization

Diastereomers were prepared by redissolving the residue in 40 μl of borate buffer (1 *M*, pH 8.5) and 50 μl of Flec reagent (1 m*M* in acetone). After 30 min at room temperature, 100 μl of 30 m*M* hydroxyproline were added. After 2 min the mixture was vortex-mixed with 300 μl of *n*-pentane for 15 s to remove excess reagent. After centrifugation (10 min, 3015 *g* at 4°C) the *n*-pentane phase was discarded. The aqueous mixture was extracted by shaking for 10 min with 3 ml of dichloromethane and centrifuged (10 min, 3015 *g* at 4°C). The organic phase was transferred to a conical glass tube and evaporated at room temperature under nitrogen. The residue was dissolved in 50 μl of the mobile phase.

Calibration curves

Aliquots of the racemic atenolol working solution were added to 100 μl of blank rat plasma, in the range 10–400 ng of free base per ml of plasma for each enantiomer. Samples were extracted and derivatized as described above. Calibration curves based on peak-height ratios (atenolol enantiomer *versus* the more slowly eluting practolol enantiomer) against the concentrations of each enantiomer were constructed using six different concentrations. The data were then subjected to linear regression analysis.

Extraction efficiency

Plasma samples ($n = 5$) containing 20–500 ng/ml racemic atenolol were extracted according to the above extraction method without addition of internal standard. Before evaporation of the extraction solvent, the internal standard was added. The samples were further analysed according to the HPLC procedure for racemic atenolol [6]. The peak-height ratios of the racemic non-derivatized atenolol *versus* the internal standard were then compared with the peak-height ratios obtained from reference samples with comparable concentrations, added to the extraction solvent and evaporated.

Accuracy and precision

Plasma samples spiked with 25, 50 and 125 ng/ml of each of the atenolol enantiomers were subjected to the assay procedure, and the concentrations of the individual enantiomers were determined from the calibration curves intra-day and inter-day.

Application

The assay was used to measure the atenolol enantiomers in male Wistar rats after intravenous administration of 2 mg/kg racemic atenolol. Blood samples were obtained as a function of time, and plasma was stored at −20°C until assay.

RESULTS AND DISCUSSION

Chromatography

Chromatograms of extracts from blank plasma, blank plasma spiked with 2 ng of both *R*-(+)- and *S*-(−)-atenolol and plasma obtained 15 min after administration of 2 mg/kg racemic atenolol are shown in Fig. 2. The chiral derivatization of atenolol and practolol results in the exclusive formation of two pairs of equally sized peaks. The first pair of peaks, with retention times of 5.95 min (*S*-(−)-atenolol) and 6.55 min (*R*-(+)-atenolol), corresponds to the diastereomeric derivatives of atenolol. The elution order of the individual enantiomers was checked with a sample of pure *S*-(−)-atenolol enantiomer. The second pair of peaks, with retention times of 7.80 and 8.64 min, results from the derivatization of the practolol enantiomers.

Extraction efficiency

The extraction efficiency for atenolol, using 3% (v/v) heptafluoro-1-butanol in methylene chloride at pH 12, was 82.0 ± 5.1% at plasma concentrations of 20–800 ng/ml. Heptafluoro-1-butanol was used as it is a strong hydrogen donor, and it has previously been shown to increase the extraction efficiency of atenolol [4]. With other extraction solvents, *n*-butanol–*n*-hexane (50:50, v/v) and dichloromethane–*n*-butanol (85:15, v/v), recoveries of less than 60% were obtained.

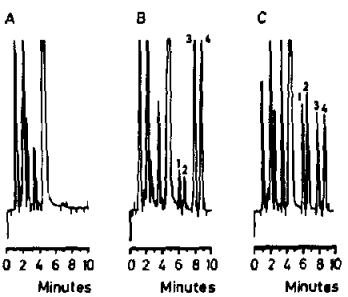

Fig. 2. Chromatograms showing resolution of the diastereomeric derivatives of *S*-(−)-atenolol (peak 1) and of *R*-(+)-atenolol (peak 2), and the diastereomeric derivatives of the practolol enantiomers (peaks 3 and 4). Extracts were obtained from (A) 100 μl of blank rat plasma, (B) 100 μl of a rat plasma spiked with 2 ng of each enantiomer and (C) 50 μl of a rat sample 15 min after intravenous administration of 2 mg/kg racemic atenolol. The calculated *S*-(−)-atenolol and *R*-(+)-atenolol concentrations were 541.6 and 636.5 ng/ml, respectively. Plasma samples (B) and (C) were spiked with 500 ng of the internal standard practolol.

Derivatization efficiency

To optimize the derivatization, 1 *M* borate buffers with different pH values (7–9.5) were tested, and a pH of 8.5 gave the most satisfactory result. At room temperature, the reaction yield for both atenolol diastereomers and practolol diastereomers increased with time and reached a maximum after *ca.* 30 min. Using these optimal conditions, the derivatization yield, based on residual analyses of racemic atenolol, was 97.1 ± 2.1% (mean ± S.D., $n = 5$).

Derivatization procedure

Extraction with pentane and reaction with the hydrophilic amino acid hydroxyproline can both be used to remove excess Flec reagent [5]. Extraction with pentane was more efficient as it also eliminates late-eluting peaks. Addition of hydroxyproline to the reaction mixture diminishes the loss of practolol diastereomers during the pentane extraction.

Internal standard

Practolol was used as internal standard. Tocainide was also evaluated for its suitability as internal standard but although the derivatization was apparently successful, it could not be used as internal standard. There was a loss of tocainide diastereomers of more than 90% during the extraction step with *n*-pentane to remove excess reagent. The loss of practolol diastereomers was 30% (± 1%, $n = 5$) under the conditions used, and there was no loss of atenolol diastereomers.

Calibration curves

The calibration curves for both *R*-(+)- and *S*-(−)-atenolol were linear over the range evaluated (10–400 ng/ml). The typical mean (± S.D.) equations ($n = 10$) describing this relationship were $y = -0.00931$ (± 0.02174) + 0.00280 (± 0.00017) x with $r = 0.99779$ (± 0.00187) for *S*-(−)-atenolol, and $y = -0.00712$ (± 0.01484) + 0.00247 (± 0.00015) x with $r = 0.99771$ (± 0.00175) for *R*-(+)-atenolol, where y and x are peak-height ratios and plasma concentrations, respectively; r is the correlation coefficient.

Precision and accuracy

The intra-day coefficient of variation (C.V.) and accuracy ranged from 3.9 to 8.1% and from 96.4 to 102.6%, respectively, for concentrations between 25 and 125 ng/ml for each enantiomer (Table I). The inter-day C.V. and accuracy ranged from 5.0 to 12.8% and from 97.5 to 101.4%, respectively, for the same concentration range.

Quantitation limit

Based on a C.V. of less than 15%, and using 100 μl of plasma, the quantitation limit was 10 ng/ml for each enantiomer.

TABLE I

ACCURACY AND PRECISION FOR THE ASSAY OF *R*-(+)-ATENOLOL AND *S*-(−)-ATENOLOL

Compound	Concentration (ng/ml)	*n*	Mean result (%)	C.V. (%)
Intra-day				
R-(+)-Atenolol	25	5	102.6	7.3
	50	5	98.8	6.5
	125	5	97.2	4.3
S-(−)-Atenolol	25	5	98.7	8.1
	50	5	102.5	3.9
	125	5	96.4	4.7
Inter-day				
R-(+)-Atenolol	25	10	101.4	9.9
	50	10	100.8	6.8
	125	9	97.8	6.1
S-(−)-Atenolol	25	10	98.4	12.8
	50	10	99.9	5.0
	125	9	97.5	6.9

Application

A typical plasma concentration–time curve for *S*-(−)- and *R*-(+)-atenolol after intravenous administration of 2 mg/kg racemic atenolol to a rat is shown in Fig. 3. The plasma concentrations of *R*-(+)- are higher than those of *S*-(−)-atenolol, but the differences are rather small. Similar results were found in humans and rats [7].

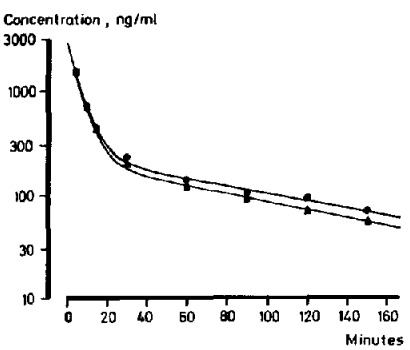

Fig. 3. Plasma concentration–time curves of *R*-(+)-atenolol (●) and *S*-(−)-atenolol (▲) after intravenous administration of 2 mg/kg racemic atenolol to a rat. The curve is fitted according to a two-compartmental model.

CONCLUSION

The column liquid chromatographic method reported is highly sensitive and selective for the determination of atenolol enantiomers in rat plasma. The applicability of the assay for pharmacokinetic studies of atenolol enantiomers in the rat is demonstrated.

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