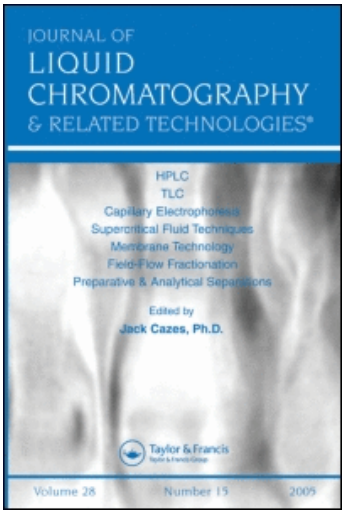

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High-Performance Liquid Chromatographic Assay for the Derivatized Enantiomers of Atenolol in Whole Blood

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DERIVATIZED ENANTIOMERS OF ATENOLOL IN WHOLE BLOOD

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ABSTRACT

A stereospecific high-performance liquid chromatographic (HPLC) method for the analysis of atenolol in human whole blood has been developed. The analytes were extracted from alkalinized plasma with ethyl acetate and derivatized with (-)-menthyl chloroformate (MCF). The resulting diastereomers were separated on a Hypersil ODS column with acetonitrile/water/methanol (43/32/25, V:V:V) as mobile phase and detected by fluorescence (230/305nm). Retention times for the diastereomeric derivatives of S(-)- and R(+)- atenolol were 9.8 and 11.0 minutes, respectively, with a resolution greater than 1.5. Standard curves were linear from 25.0 to 400 ng/mL for each enantiomer and exhibited correlation coefficients of 0.9975 or better (n=6). Inter-assay variability was less than 7.5% for each enantiomer. Furthermore, the assay was suitable for measuring the concentrations of S(-)- and R(+)-atenolol in human whole blood after a single 100 mg oral dose of the racemate.

INTRODUCTION

The use of β-blocking drugs in the management of cardiovascular disorders is well established. Atenolol,

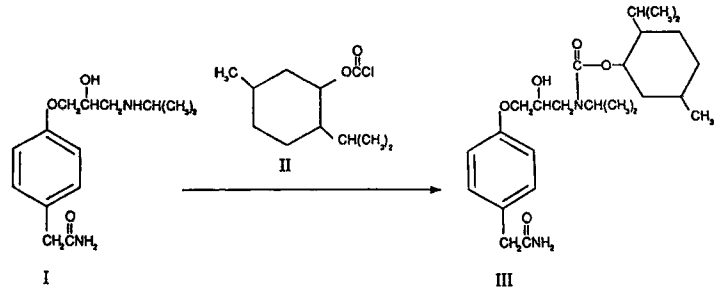

FIGURE 1. Structure of (I) atenolol, (II) MCF, and (III) derivatized atenolol.

2-[4-(2-hydroxy-3-isopropylaminopropoxy) phenyl] acetamide (Figure 1), is a cardioselective β_1-adrenergic receptor blocking agent prescribed for the treatment of angina pectoris and hypertension[1].

Atenolol has a chiral center and as such, two enantiomeric forms exist, namely S(-)- and R(+)-atenolol. In a general case, the desired pharmacological activity often resides in only one of the enantiomers, while the other could possess entirely different or unwanted pharmacological or toxicological activity. Thus, the availability of a chiral assay for atenolol in whole blood may provide further pharmacokinetic and pharmacodynamic information.

Although the separation of enantiomers is not possible with conventional reversed-phase HPLC, the formation of diastereomers through derivatization can enable their separation. The derivatization agent (-)-menthyl chloroformate can react with alcohols and amines to form a carbonate or carbamate, respectively. In the case of atenolol, MCF reacts only with the amine to form a carbamate[2].

To our knowledge, no bioanalytical assays have been reported for the enantiomeric determination of atenolol in whole blood. However, methods exist for plasma and urine[2-7]. The analysis of atenolol in whole blood could be required if a limited blood draw is necessary and/or it is given in combination with a drug that could only be analyzed in whole blood. Consequently, this prompted the development of a method in whole blood which is specific, sensitive and robust.

This manuscript describes a reversed-phase HPLC assay for the enantiomeric separation of atenolol in human whole blood. The method is linear over the concentration range of 25.0 to 400 ng/mL. The upper limit of 400 ng/mL for each enantiomer was selected on the basis of its C_{max} of 640 ng/mL (racemate) in plasma[8]. Furthermore, this procedure can be applied to ascertain the pharmacokinetics of a single 100 mg oral dose of Tenormin® in humans over a 24 hour period.

EXPERIMENTAL

Reagents and Chemicals

S(-)- and R(+)-atenolol and (-)-menthyl chloroformate were purchased from Aldrich (Milwaukee, WI, USA). Racemic atenolol, methoxamine hydrochloride and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol, acetonitrile and glass distilled ethyl acetate were purchased from Caledon (Georgetown, ON, Canada). HPLC grade phosphoric acid 85%, isopropanol, potassium phosphate monobasic, triethylamine and sodium

carbonate were purchased from Fisher Scientific (Montreal, QC, Canada). The water was deionized Type 1, reagent grade (Millipore, Ville St. Laurent, QC, Canada). All reagents were used without further purification.

Instrument and Chromatographic Conditions

The chromatographic system consisted of a Waters 590 pump (Waters Associates, Milford, MA, USA), a Varian 9090 autosampler (Varian, Ville St. Laurent, QC, Canada) and a Shimadzu RF-535 fluorescence detector (RP Instruments, Montreal QC, Canada). A stainless-steel column (15 cm x 4.6 mm i.d.) was packed with Hypersil ODS, particle size 5 micron (prepared in-house). The column was maintained at ambient temperature. The diastereomeric derivatives of atenolol were detected by fluorescence with excitation and emission wavelengths of 230 and 305 nm, respectively. The mobile phase consisted of water/acetonitrile/methanol (32/43/25, V:V:V), and was delivered at a flow rate of 1.2 mL/minute. Under these conditions, the retention times of S(-)- and R(+)-atenolol and the internal standard were 9.8, 11.0 and 14.0 minutes, respectively.

Biological Samples

Whole blood samples were collected from healthy volunteers after receiving a single 100 mg oral dose of Tenormin^{R}. Blood was drawn into evacuated EDTA collection tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA) and stored at -15°C until analyzed.

Preparation of Standards

A stock solution of atenolol containing 0.50 mg/mL of each enantiomer was prepared in methanol. Appropriate dilutions of the stock were made with deionized water and used to prepare spiked whole blood standards at S(-) and R(+) concentrations of 25.0, 50.0, 100, 200 and 400 ng/mL. Spiked whole blood quality control samples (QCs) were prepared at final concentrations of 60.0, 150 and 300 ng/mL. Individual 1.0 mL aliquots of spiked whole blood were stored in 13 x 100 mm borosilicate tubes at -15°C. A stock internal standard solution of methoxamine (free base) was prepared at 1.0 mg/mL and diluted to 2.0 µg/mL with 0.1 N sodium hydroxide. All stock solutions were stable for at least one month at -15°C.

Whole Blood Extraction

Aliquots of human whole blood (1.0 mL) were added to 13 x 100 mm borosilicate tubes and alkalinized with 1.0 mL of 0.1 N NaOH containing 2.5 µg/mL internal standard and vortexed. Ethyl acetate (5 mL) was added and the tubes were shaken for 15 minutes at low speed (230 ± 30 oscillations/minute) on a reciprocating shaker. Following centrifugation for 15 minutes at ca. 2000 g, the organic layer was transferred to a clean 13 x 100 mm disposable borosilicate tube. An additional 3 mL of ethyl acetate was added to the aqueous layer and re-extracted as above. The organic layers were combined and evaporated to dryness at 37°C under a gentle stream of nitrogen.

Chiral Derivatization

After evaporation, 50 µL of 0.1 N NaOH was added to the residue and vortexed briefly, then 200 µL of 0.2 M (-)-menthyl

chloroformate prepared in acetonitrile was added and vortexed for 30 seconds. The tubes were left at room temperature 10 minutes and 50 µL was injected onto the liquid chromatograph under the previously stated conditions. Once formed, the diastereomers were stable for at least 36 hours at room temperature.

The pure enantiomers of atenolol were chromatographed separately following derivatization to assess their elution order.

Derivatization Yields

To ascertain the derivatization yields of S(-)- and R(+)-atenolol, the absolute peak heights of the underivatized to derivatized analytes were compared. The underivatized analytes were measured using an achiral method with an absolute detection limit of 10.0 ng/mL for the racemate[9]. The mobile phase was modified to 2% acetonitrile, 8% methanol in 0.05 M phosphate-triethylamine, pH 3.3 and the fluorescence detector was set at 230 and 300 nm for excitation and emission, respectively.

Extraction Yields

Spiked whole blood (n=8) at concentrations of 80 and 360 ng/mL were extracted as described except exactly 3.0 mL of the organic phase was removed with each extraction (6 mL total). The combined organic layers were evaporated to dryness and reconstituted in the mobile phase. The peak heights of the racemic non-derivatized atenolol versus unextracted equivalent concentrations were compared under identical conditions, using the achiral method described above.

Data Acquisition

The peak height ratios of the diastereomeric derivatives of atenolol were measured with a Spectra-Physics model 4270 integrator and down-loaded to Chrom-Station (Spectra-Physics Inc., Mountain View, CA, USA). The chromatographic data were automatically processed for the peak height ratios of each enantiomer and fitted to a weighted (1/C) linear regression.

Accuracy and Precision

To assess the accuracy and precision of the assay, spiked human whole blood QC samples were prepared at S(-) and R(+)-atenolol concentrations of 60.0, 150 and 300 ng/mL. To determine accuracy, the concentration of individual enantiomers in the QC samples (n=11-12) were calculated using a standard curve. The percent difference between the measured and added concentrations was considered the accuracy of the assay. Precision was estimated by calculating the inter-assay coefficient of variation (CV).

RESULTS

Linearity and Quantitation Limits

Linear response in peak height ratios for S(-)- and R(+)-atenolol to internal standard over the concentration range of 25.0 to 400 ng/mL was observed. The correlation coefficients for the diastereomeric derivatives were 0.9975 or better (n=6). The internal standard also undergoes derivatization with MCF and results in two peaks. The first peak was arbitrarily chosen for quantitation purposes.

Typical chromatograms obtained from extracted whole blood blanks and extracted calibration samples at 25.0 and 400 ng/mL are illustrated in Figures 2(a-c). The retention times of S(-)- and R(+)-atenolol and internal standard were 9.8, 11.0 and 14.0 minutes, respectively, with an overall chromatographic run time of 18 minutes. The resolution (R_s) between the enantiomers of atenolol was 1.6.

A minimum signal-to-noise ratio (S/N) of 4:1 was obtained with the lowest standard, allowing a quantitation limit of approximately 12.5 ng/mL with a S/N of 2:1. Thus, the limit of quantitation used (25.0 ng/mL) is higher than the absolute limit of the assay.

Specificity

Human whole blood was collected from healthy donors and screened for interference at the retention times of each enantiomers and internal standard. No significant interference has been observed in drug free blood samples.

Precision and Accuracy

The inter-day precision and accuracy was assessed by the repeated analysis of whole blood specimens containing different concentrations of S(-)- and R(-)-atenolol (Table 1). Two samples at each QC concentration, low, medium and high, together with a calibration curve were run as a single batch. To be regarded as a separate batch, the entire sample processing must take place in a time domain completely separate from one another. At spiked blood

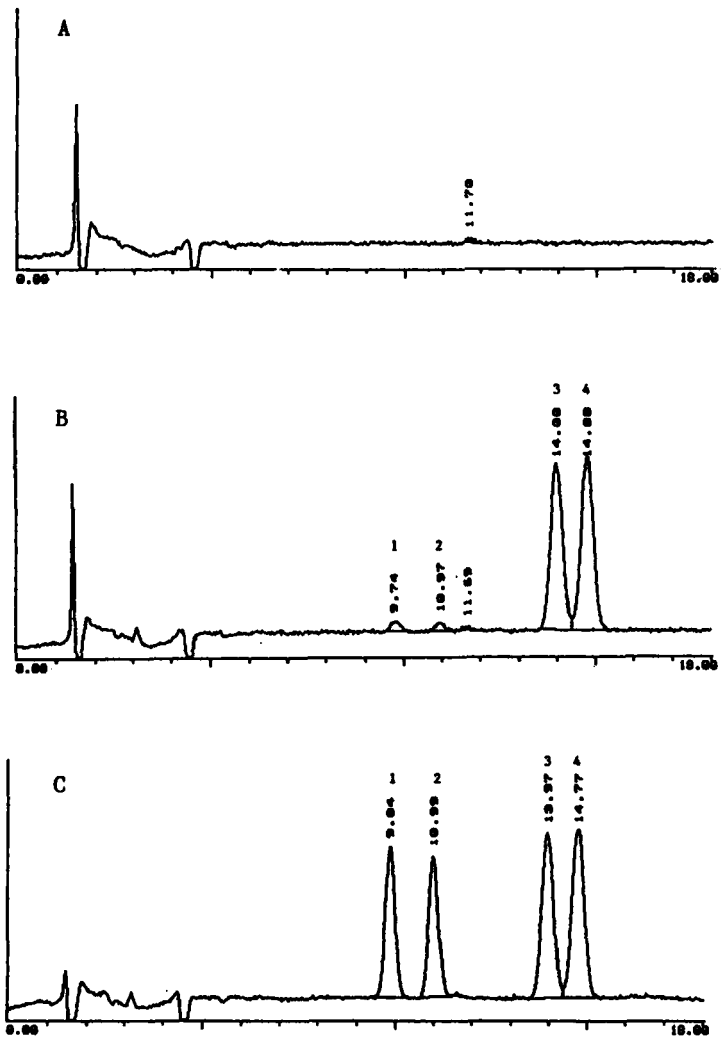

FIGURE 2. Chromatograms of (A) whole blood blank, (B) whole blood spiked with 25.0 ng/mL of each atenolol enantiomer and, (C) whole blood spiked with 400 ng/mL of each atenolol enantiomer. Peak identification: 1, S(-)-atenolol; 2, R(+)-atenolol; 3 and 4, internal standard.

TABLE 1

Inter-Day Accuracy and Precision

Atenolol Concentration ng/mL							
Added	n	Measured (mean±SD) S(−)	R(+)	Accuracy S(−)	R(+)	Precision S(−)	R(+)	S/R Ratio
STD 25.0	8	25.2 ± 1.7	25.4 ± 1.4	100.8	101.6	6.9	5.3	0.99
STD 50.0	8	49.6 ± 3.2	49.6 ± 2.0	99.2	99.2	6.4	4.1	1.00
STD 100	8	101 ± 5.3	99.9 ± 4.2	101.0	99.9	5.2	4.2	1.01
STD 200	8	198 ± 5.5	197 ± 5.3	99.0	98.5	2.8	2.7	1.01
STD 400	7	402 ± 6.3	403 ± 7.9	100.5	100.8	1.6	2.0	1.00
QC 60.0	16	58.0 ± 2.3	57.5 ± 3.0	96.7	95.8	4.0	5.2	1.01
QC 150	15	146 ± 6.4	144 ± 5.6	97.3	96.0	4.3	3.9	1.01
QC 300	16	289 ± 20.5	288 ± 21.3	96.3	96.0	7.1	7.4	1.00

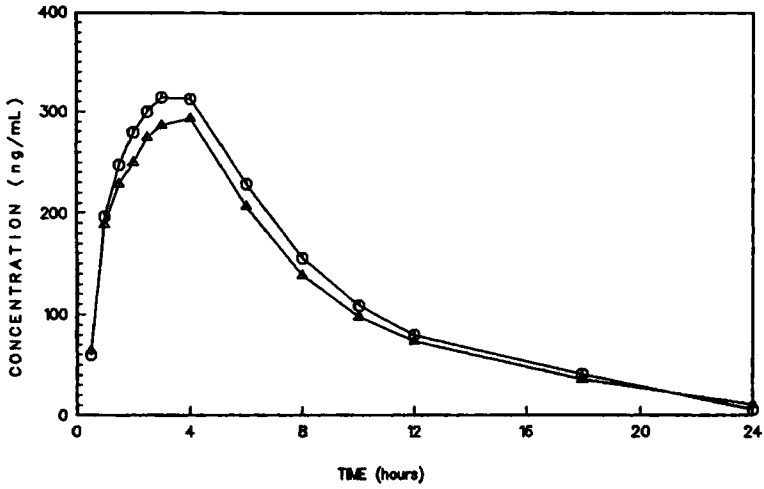

FIGURE 3. Representative whole blood concentration-time profile of S(-)-atenolol (triangle) and R(+)-atenolol (circle) of a subject after a single 100 mg oral dose of Tenormin®.

concentrations of 60.0, 150 and 300 ng/mL for each enantiomer, the method yields coefficients of variation ranging from 3.9 to 7.4%. The mean concentrations obtained from the calibration curve ranged from 98.7 to 101.5% of the nominal concentration of S(-)- and R(+)-atenolol.

Application

Human whole blood was collected from healthy volunteers in Vacutainers containing EDTA prior to dosing and at 13 subsequent time points. Following collection, the samples were stored at -15°C until analyzed. All samples were analyzed by the method presented

TABLE 2

Normalized Peak Heights for the Diastereomeric Derivatives of Atenolol as a Function of NaOH Volume

	0.1 N NaOH Volume (µL)				
	0	5	10	20	50
S(-)-atenolol					
mean*	31.7	77.0	87.3	97.0	99.3
SD	13.65	10.82	6.03	1.00	0.58
CV(%)	43.1	14.0	6.9	1.0	0.6
R(+)-atenolol					
mean*	31.0	76.3	85.3	97.3	98.0
SD	12.29	10.50	5.69	1.53	1.73
CV(%)	39.6	13.8	6.7	1.6	1.8

* n=3

here. A typical concentration-time profile after a 100 mg oral dose of Tenormin^{R} is depicted in Figure 3.

The assay allows the quantitation of whole blood levels of atenolol for at least 24 hours following a single 100 mg oral dose of Tenormin^{R} and permits complete characterization of the resulting whole blood profile.

Discussion

Various organic solvents including isopropanol/pentane, n-butyl chloride, isopropanol/hexane, dichloromethane and ethyl acetate were investigated for the extractibility of atenolol. Ethyl acetate proved to be the solvent of choice with extraction yields

(mean ± SD, n=8) of 93.5 ± 5.1% and 91.4 ± 2.4% for 80.0 and 360 ng/mL atenolol from 1.0 mL of spiked whole blood.

In addition to MCF, the chiral derivatizing agent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) was also investigated since it has been used to separate the enantiomers of several β-blockers in solution[10]. However, using the derivatization schemes investigated, MCF was found to be more sensitive than TAGIT so consequently, the former was used.

The effect of diastereomeric derivatization was investigated as a function of NaOH volume. As depicted in Table 2, 50 µL of 0.1 N NaOH was optimal. A volume greater than 50 µL resulted in poor chromatography.

In whole blood, the derivatization yields of S(-)- and R(+)-atenolol were determined to be 100%, since no underivatized drug was observed on the achiral system. Also, the derivatization reaction was not stereoselective with each diastereomer exhibiting similar peak areas.

In conclusion, the chiral method described herein is sensitive and rapid for the enantiomeric analysis of atenolol in human whole blood. Furthermore, baseline resolution of the stereoisomers is achieved within 18 minutes making this assay suitable where high sample throughput is necessary.

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