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Note

High-performance liquid chromatographic determination of the enantiomers of β-adrenoceptor blocking agents in biological fluids

II. Studies with atenolol

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Atenolol, a cardioselective β-adrenoceptor blocking agent [1], is used clinically for the treatment of angina pectoris and hypertension. Like most β-adrenoceptor blocking agents, atenolol is marketed as a racemic mixture of two enantiomers. Because the β-adrenoceptor blocking activity appears to reside in the *S*-(−)-enantiomer, it is desirable clinically to measure the concentrations of this enantiomer in biological fluids such as plasma [2]. To date, a number of procedures have been developed to analyze the total concentrations of racemic atenolol in plasma and urine. These procedures have employed thin-layer chromatography [3], gas chromatography [4], and high-performance liquid chromatography with either ultraviolet or fluorometric detection [5–13]. To our knowledge, no procedure for analysis of the individual enantiomers of atenolol has been reported.

In this manuscript, an analytical procedure for the individual enantiomers of atenolol in plasma and urine is presented. The procedure involves extraction of atenolol and an internal standard, (±)-4-methylpropranolol, from biological fluids followed by derivatization at room temperature with *S*-(−)-α-methylbenzyl isocyanate. The resultant diastereomers are then separated by reversed-phase high-performance liquid chromatography (HPLC) and detected by fluorometry. The procedure is reproducible and sufficiently sensitive to allow for detection of the individual enantiomers following therapeutic doses of the racemate. Recently the procedure was used to ascertain the stereoselective pharmacokinetics of atenolol following a single oral dose of racemic atenolol [14].

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EXPERIMENTAL

Chemicals and reagents

S-(−)-α-methylbenzyl isocyanate, 99% pure (Aldrich, Milwaukee, WI, U.S.A.), racemic atenolol (Sigma, St. Louis, MO, U.S.A.), ethyl acetate (Fisher Scientific, Springfield, NJ, U.S.A.), and chloroform, HPLC grade (Fisher Scientific) were used without further purification. Methanol (Fisher Scientific) was HPLC grade. The internal standard, (±)-4-methylpropranolol, was a gift from Dr. Wendel L. Nelson and the enantiomers of atenolol were generously supplied by Dr. Hildegard Spahn for use in identification of the peaks.

Two working solutions of racemic atenolol for calibration of the assay were prepared daily by diluting the stock solution of racemic atenolol in distilled water to concentrations of 10 and 50 mg/l. The working solution of internal standard was prepared by diluting an appropriate volume of the stock solution of internal standard, (±)-4-methylpropranolol in a 1:1 mixture of acetonitrile–distilled water to achieve a concentration of 1 mg/l. The stock solutions of racemic atenolol (1.0 mg/ml) and (±)-4-methylpropranolol (0.1 mg/ml) were prepared in distilled water and a 1:1 mixture of acetonitrile and distilled water, respectively, and stored at −20°. Daily, 2 μl of *S*-(−)-α-methylbenzyl isocyanate were dissolved in 10 ml of chloroform for use as the derivatizing solution.

Sample preparation

Plasma (1 ml) or urine (0.5 ml) was placed in a 150×20 mm disposable borosilicate culture tube. A 0.5-ml volume of the solution of internal standard, 50 μl of 1 *M* sodium hydroxide and 200 μl of a saturated solution of sodium chloride were added, and the mixture was vortexed for 30 s. To the resulting mixture 3 ml of ethyl acetate were added and the two-phased mixture was vortexed for 1 min and then centrifuged at 1800 *g* for 15 min. A 2-ml volume of the organic phase was transferred to a 150×20 mm disposable borosilicate culture tube and evaporated to dryness under a stream of nitrogen at room temperature. A 0.5-ml volume of the derivatizing solution was added, the mixture vortexed and evaporated to dryness under a nitrogen stream. The total time for reaction including the evaporation step was less than 5 min. Preliminary studies with the individual atenolol enantiomers showed that this time was sufficiently long for complete derivatization. The samples were reconstituted into 0.5 ml of mobile phase and 50 μl were injected onto the column.

Chromatography

The HPLC system used in this study included a Beckman Model 110A pump (Beckman Instruments, Fullerton, CA, U.S.A.), an Altex Model 210 manual injector (Beckman Instruments), and a variable-wavelength fluorometer (Kratos Model GM 970 monochromator, Kratos FS 970 LC fluorometer, Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) with the excitation wavelength set at 216 nm and no emission cut-off filter included. The recorder was an Omniscribe Series D-5000 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). The column was a reversed-phase C_{18} column (Altex Ultrasphere ODS 5 μm, 15

cm × 4.6 mm I.D., Beckman Instruments, Berkeley, CA, U.S.A.). The mobile phase was methanol–water (50:50). Mobile phase was filtered under vacuum through millipore filters prior to use. Flow-rate was 1.2 ml/min which produced a column inlet pressure of 6.89 MPa. The detector range was adjusted to 0.02 μA.

Characteristics of the assay

Calibration. Calibration of the assay was performed by analyzing 1.0-ml aliquots of blank plasma to which 100–3000 ng of racemic atenolol had been added or 0.5-ml aliquots of blank urine to which 10–6000 ng of racemic atenolol had been added. Five replicates were determined at each concentration. Nine concentrations were used for each calibration curve. For each concentration, the peak height of the *R*-(+)- or *S*-(−)-atenolol derivative was measured and divided by the peak height of the internal standard to obtain a peak-height ratio. Linear least-squares regression analysis was used to obtain a slope and intercept which were then used to determine the concentrations of each enantiomer in the unknown samples.

Reproducibility and precision. Peak-height ratios were measured and the coefficient of variation (C.V.) was calculated at each concentration. Reproducibility measurements were based upon five replicates of peak-height ratios obtained in a single day. Slopes of the calibration curves obtained from both urine and plasma were measured on five different days and the variation of the slope was used to measure the inter-day variance. To obtain the precision, the concentrations of each enantiomer were calculated from the peak-height ratios and the calibration curves from urine and plasma.

Recovery from extraction. Aliquots (1 ml) of plasma and urine were spiked with 500 or 2000 ng of racemic atenolol. After the samples were extracted, derivatized, and chromatographed as previously described, the peak height of each atenolol diastereomer was compared to the peak height obtained when the same amount of each enantiomer in chloroform was treated with 0.5 ml of the derivatizing solution. The chloroform was evaporated under a nitrogen stream, the residue reconstitued into 500 μl of mobile phase, and 50 μl were injected into the chromatograph. Four replicates from plasma and urine were determined at each concentration. The percentage recovery from the extraction was calculated as the peak height of the enantiomer derivative which had been derived after extraction from the biologic fluid divided by the peak height of the enantiomer derivative which had been derived from enantiomers which had not undergone extraction. Recovery was corrected for the loss of enantiomer that occurs by taking 2 of 3 ml of the ethyl acetate phase.

RESULTS

Chromatography and identification of peaks

Chromatograms from typical plasma and urine samples are shown in Fig. 1. As shown, the retention times of the atenolol diastereomers were 16 and 18 min with baseline separation. The internal standard had a retention time of 20 min. When individual enantiomers were analyzed, the retention time corresponding to the

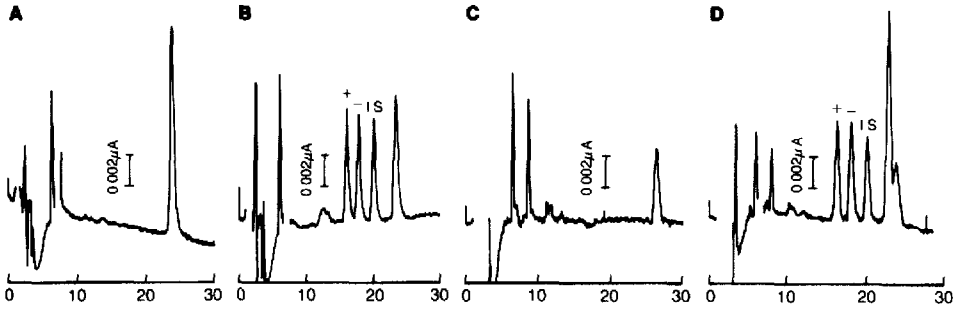


Fig. 1. Chromatograms obtained after extraction of racemic atenolol followed by derivatization with (*S*)-(−)-(α)-methylbenzyl isocyanate. (A) Blank plasma sample; (B) plasma sample initially containing 500 ng/ml of each atenolol enantiomer; (C) blank urine sample; (D) urine sample initially containing 1000 ng/ml of each atenolol enantiomer.

TABLE I

CALIBRATION CURVES OF THE ATENOLOL ENANTIOMERS

Sample	Slope[a]	Intercept[b]	Correlation coefficient
R-(+)-Atenolol[c]	0.0020 ± 0.0001	0.013 ± 0.016	0.999
S-(−)-Atenolol[c]	0.0020 ± 0.0001	0.014 ± 0.018	0.999
R-(+)-Atenolol[d]	0.0021 ± 0.0001	0.056 ± 0.021	0.999
S-(−)-Atenolol[d]	0.0021 ± 0.0001	0.048 ± 0.031	0.999

[a]Values of the slope are the mean ± S D. from curves obtained on five different days.
[b]Values are not significantly different from 0.
[c]Plasma samples.
[d]Urine samples.

R-(+)-enantiomer derivative was 16 min and the retention time associated with the *S*-(−)-enantiomer derivative was 18 min.

Characteristics of the assay

Calibration. Peak-height ratios of the *R*-(+)- and *S*-(−)-atenolol diastereomers correlated linearly with concentration in the range used. Correlation coefficients of the lines were all greater than 0.999 (Table I). The limit of detection, defined as four times baseline noise, was 50 ng/ml.

Reproducibility and precision. Shown in Table II are data demonstrating the reproducibility and precision of the assay. For plasma, the C.V.s of the two derivatives ranged from 2 to 6%. For urine, the C.V.s of the two derivatives ranged from 2 to 9%. The lower C.V.s were obtained at the higher concentrations in both plasma and urine. With respect to precision, calculated concentrations were within 7% of the actual concentrations initially present in plasma. When the enantiomers were initially present in urine, the precision of the assay was excellent except at the lowest concentration (200 ng/ml). At this concentration, the calcu-

TABLE II

REPRODUCIBILITY AND PRECISION OF THE ASSAY FOR *R*-(+)- AND *S*-(−)-ATENOLOL IN PLASMA AND URINE

Value in parentheses represents coefficient of variation (%).

Expected concentration[a] (ng/ml)	Calculated concentration[b] (ng/ml)	
	R-(+)-Atenolol	*S*-(−)-Atenolol
Plasma		
100	93.6[b] (5.0)[c]	93.2 (5.0)
250	268 (6.0)	248 (4.4)
1000	1000 (2.5)	973 (1.9)
Urine		
200	165 (9.0)	173 (5.6)
500	517 (7.3)	506 (7.8)
2000	2060 (2.1)	1960 (2.3)

[a]Concentration of each atenolol enantiomer initially present in plasma or urine.
[b]Mean concentrations of five determinations of each atenolol enantiomer obtained on a single day.

lated concentrations of the *R*-(+)- and *S*-(−)-enantiomer were within 20% of the actual concentration.

Recovery from extraction. At an initial concentration of 250 ng/ml, the recovery of *R*-(+)-atenolol from plasma was 66.8 ± 2.2% and of *S*-(−)-atenolol 64.4 ± 2.7%. In urine, the recovery of the individual enantiomers was somewhat lower and averaged 57.0 ± 1.8% for *R*-(+)-atenolol and 57.2 ± 6.7% for *S*-(−)-atenolol. Recoveries were comparable at the higher concentration. At 1000 ng/ml, the recovery from plasma of *R*-(+)- and *S*-(−)-atenolol, respectively, was 62.3 ± 5.1 and 60.8 ± 5.0%. From urine, the recoveries were 52.6 ± 3.5% for the *R*-(+)-enantiomer and 52.9 ± 5.1% for the *S*-(−)-enantiomer.

DISCUSSION

β-Adrenoceptor blocking agents such as atenolol are usually marketed clinically as racemic mixtures of two enantiomers. In general the *S*-(−)-enantiomer is considerably more potent than the *R*-(+)-enantiomer in terms of its β-adrenoceptor blocking activity [2]. Because enantiomers often differ in their pharmacokinetic properties, concentrations of the racemic mixture in biologic fluids may not reflect the concentrations of the active enantiomer. Consequently, it is desirable clinically and in pharmacodynamic studies to use stereospecific analytical procedures to measure the concentrations of each enantiomer following administration of the racemate [2]. Although a number of analytical procedures

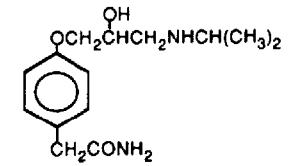
have been developed for racemic atenolol [3–13], there have been no procedures reported for the analysis of the individual enantiomers in the presence of the other enantiomer.

Several stereospecific analytical procedures for other β-adrenoceptor blocking agents, particularly propranolol, have been described [15–23]. Most of the procedures have involved the formation of diastereomers of racemic propranolol with optically pure chiral reagents followed by separation of the diastereomers with reversed-phase chromatography. N-Trifluoroacetyl-(−)-prolyl chloride [13], *tert.*-butoxycarbonyl-L-leucine anhydride [18], and *S*-(−)-α-methylbenzyl isocyanate have been used as derivatizing agents [19–21]. Of these, *S*-(−)-α-methylbenzyl isocyanate appears to be the most useful because it is commercially available, stable, and can be used at room temperature. Preliminary data have suggested that *R*-(−)-1-(1-naphthyl)ethyl isocyanate may also be useful for analyzing enantiomers of β-adrenoceptor blocking agents in biological fluids [22].

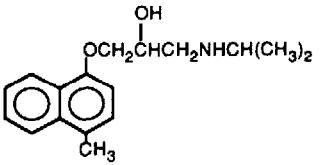
Recently, using *S*-(−)-α-methylbenzyl isocyanate as a derivatizing agent, we developed an HPLC procedure for the separation and detection of the individual enantiomers of the β-adrenoceptor blocking agent, pindolol, in biologic fluids [21]. The procedure was sensitive, specific, and reproducible, and was used in a pharmacokinetic/pharmacodynamic study to analyze D- and L-pindolol following therapeutic doses of the racemic mixture [24]. However, the procedure did not incorporate an internal standard and involved multiple extraction steps.

In this study, *S*-(−)-α-methylbenzyl isocyanate was used to form diastereomers of atenolol. The diastereomers formed are likely to be urea derivatives as depicted in Fig. 2. Previously, using mass spectrometry, it has been shown that propranolol [19] and pindolol [21] form urea diastereomers with *S*-(−)-α-methylbenzyl isocyanate. Unlike the previous procedure for the analysis of the enantiomers of pindolol, an internal standard, (±)-4-methylpropranolol, was incorporated into this procedure to determine the enantiomers of atenolol. Criteria for the selection of the internal standard were that the compound should interact with *S*-(−)-α-methylbenzyl isocyanate, fluoresce, and chromatograph similarly to the atenolol diastereomers. (±)-4-Methylpropranolol fulfilled these criteria. Under the chromatographic conditions, (±)-4-methylpropranolol formed two diastereomers which were completely resolved. The retention times of the diastereomers were 20 and 23 min; however, the diastereomer with the later retention time resulted in a variable peak height (Fig. 1) and co-chromatographed with an interfering peak present in biologic fluids (Fig. 1). Thus, the diastereomer with the earlier retention time was used and provided an excellent internal standard as indicated by the reproducibility of the assay.

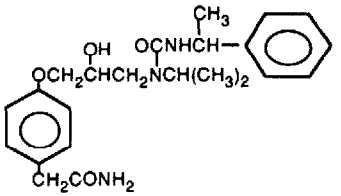
The assay of both enantiomers of atenolol from either plasma or urine was highly reproducible and linear over the concentrations tested. The C.V.s at all concentrations studied were less than 10% for both enantiomers (Table II). The correlation coefficients of the calibration curves for *S*-(−)- and *R*-(+)-atenolol in both plasma and urine were all greater than 0.999. Inter-day variability was low as indicated by the small standard deviations of the slopes of the calibration curves obtained on five separate days (Table I). Precision was excellent except at very low concentrations of the enantiomers in urine in which the actual con-



Atenolol



4-Methyl-Propranolol



Urea Derivative

Fig. 2. Structures of atenolol, 4-methylpropranolol, and the proposed urea diastereomers formed after reaction of atenolol with (*S*)-(−)-α-methylbenzyl isocyanate.

centrations were underestimated. At 200 ng/ml the measured values of the enantiomers appeared to be between 15 and 20% lower than the actual concentrations initially present in the urine. The reason for this discrepancy is unknown. Recovery of the enantiomers present initially at 250 ng/ml in both plasma and urine was highly reproducible and averaged 65 and 57% for plasma and urine, respectively. At higher concentrations recoveries from both biologic fluids was slightly lower averaging 62 and 53% from plasma and urine, respectively. It is not known why the recovery of both enantiomers of atenolol from plasma was greater than from urine. Previously, we and others observed a substantially greater recovery of pindolol from plasma than from urine [21,25]. These data highlight the importance of calibrating the assay in the appropriate biologic fluid.

Recently, we employed this procedure in a pharmacokinetic study of atenolol in normal volunteers [14]. A single therapeutic dose of racemic atenolol (100 mg) was administered orally to six normal volunteers and the individual enantiomers were measured in both plasma and urine. The data suggest that atenolol may be absorbed stereoselectively. The study demonstrates the usefulness of this

procedure in quantitating the enantiomers of atenolol following therapeutic doses of the racemate.

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