CHROMBIO. 5086

Stereospecific high-performance liquid chromatographic assay of acebutolol in human plasma and urine

M. PIQUETTE-MILLER, R.T. FOSTER*, F.M. PASUTTO and F. JAMALI

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, T6G 2N8 (Canada)

SUMMARY

A sensitive high-performance liquid chromatographic technique is described for the separation of *R*- and *S*-acebutolol in human plasma and urine. The procedure involves derivatization with the chiral reagent *S*-(+)-1-(1-naphthyl)ethyl isocyanate. The resulting diastereoisomers are quantified using normal-phase high-performance liquid chromatography with fluorescence detection (220/389 nm). Virtual baseline separation, free from interference, was achieved (resolution factor = 1.45). Excellent linearity ($r > 0.998$) was observed throughout the range 10–500 ng/l and 2–100 mg/l in plasma and urine, respectively. Inter-assay variability was less than 5% for each enantiomer at concentrations of 10 ng/ml. This method is applicable for the determination of the pharmacokinetics, in man, of acebutolol enantiomers in plasma and urine.

INTRODUCTION

The stereospecific analysis of a number of β-adrenergic blocking drugs (β-blockers) has recently received widespread attention [1–8]. This attention stems from the fact that, with the exceptions of timolol and penbutolol, all of the β-blockers are manufactured and administered as racemic mixtures [9]. Generally, the β-blocking activity has been ascribed to the *S*-(−)-enantiomer; the *R*-(+)-enantiomer is devoid of this activity [10]. In addition to the knowledge that the enantiomers differ pharmacologically, it is also known that some of the enantiomers of various β-blockers possess quite different pharmacokinetics [3,11–13]. For these reasons, it is apparent that the disposition of the individual enantiomers must be delineated.

The β-blocker (±)-acebutolol (AC) was introduced in France and the United Kingdom in the mid 1970s [14]. It is indicated in the treatment of hypertension and ventricular arrhythmia. AC has been measured in plasma using radioimmunoassay [15] and various reversed-phase high-performance liquid chromatography (HPLC) techniques [16,17]. However, these methods did not measure individual enantiomers of AC. More recently, the enantiomers of AC have been determined in plasma using chiral derivatization techniques. These techniques have included the use of trifluoroacetyl-L-prolyl chloride (TPC) [10], although it was shown that this reagent rapidly racemizes during storage. Alternatively, either R-(+)-1-phenylethyl isocyanate (R-PEIC) [18] or S-(−)-PEIC [8] have been used to derivatize other β-blocking drugs, including AC. These methods utilized reversed-phase HPLC and fluorescence detection. The method reported by Gulaid et al. [18], however, lacked the sensitivity required to be useful clinically when low doses are administered to man (100–400 mg twice daily). The former method [8], although useful for pindolol, did not report sensitivity for AC analysis. In this paper, we report a sensitive, stereospecific HPLC assay suitable for determination of the pharmacokinetics of AC enantiomers in plasma and urine.

EXPERIMENTAL

Chemicals

The β-blockers used in this study were obtained as gifts from the respective pharmaceutical firms: racemic AC, S-(−)-AC and R-(+)-AC (Rhone-Poulenc, Essex, U.K.); internal standard (I.S.), racemic pindolol (Sandoz, Dorval, Canada); racemic atenolol (Imperial Chemical Industries, Cheshire, England); nadolol (Squibb, Montreal, Canada); toliprolol (Boehringer Ingelheim, Mannheim, F.R.G.); alprenolol (Astra, St. Albans, U.K.); bupranolol (Logeais, Issy-les-Molineaux, France); labetolol (Glaxo, Toronto, Canada); metoprolol (Ciba, Mississauga, Canada); propranolol (Ayerst, Montreal, Canada); (±)-, (+)- and (−)-sotalol (Bristol Myers, Candiac, Canada). Tocainide, an antiarrhythmic drug, was also obtained as a gift (Astra). S-(+)-1-(1-Naphthyl)ethyl isocyanate (NEIC) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Hexane, chloroform, diethyl ether, isopropyl alcohol and methanol (BDH, Toronto, Canada) were all analytical grade. Water was HPLC grade and triethylamine was analytical grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

Chromatography

Samples were mixed with a Vortex Genie 2 mixer (Fisher Scientific, Edmonton, Canada) and centrifuged with a Dynac II centrifuge (Becton Dickinson, Parsippany, NJ, U.S.A.). Evaporation of solvents utilized a Savant Speed Vac concentrator–evaporator (Emerston Instruments, Scarborough, Canada). The HPLC system consisted of a Model 590 pump and a Model 712 Wisp

autosampler (Waters, Mississauga, Canada). The detector was an Applied Biosystems Model 980 fluorescence detector (Technical Marketing Assoc., Edmonton, Canada) and was set at 220 and 389 nm for excitation and emission, respectively. A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3390A integrator was used to record the peak areas. The column wa a 25-cm stainless-steel silica column (Whatman Partisil, Clifton, NJ, U.S.A., 5 μm particle size). The mobile phase consisted of hexane–chloroform–methanol (63:35:2, v/v) and was pumped at a flow-rate of 2.0 ml/min. Sample preparation and analysis were conducted at ambient temperature (22–25°C).

Mass spectrometry

To determine the identity of the diastereoisomers, both peaks corresponding to the enantiomers of AC were collected. The collected peaks were subjected to high-resolution mass spectral analysis (AEI, MS9, Manchester, U.K.) via direct insertion probe, utilizing fast atom bombardment (FAB). The medium was glycerol; positive-ion mode.

Standard solutions

A solution of 0.1% (v/v) NEIC was prepared in chloroform. A stock solution of AC (as hydrochloride salt) was prepared in water to a final concentration of 50 mg/l of the base (solution 1). Another stock solution (for determination of extraction and derivatization yields) of AC (as hydrochloride salt) was prepared in 0.01% (v/v) triethylamine in chloroform to a concentration of 50 mg/l base (solution 2). The I.S. solution was 50 mg/l pindolol (as the base) in methanol.

Drug-free plasma and urine samples were spiked with solutions of AC to give concentrations of 10, 25, 50, 100, 200 and 500 ng/ml for plasma and 2, 5, 10, 20, 40 and 100 mg/l for urine.

Sample preparation

To 1.0 ml plasma containing AC were added 0.10 ml of I.S. solution and 0.15 ml of 1 *M* sodium hydroxide. The mixture was then extracted with 5 ml of diethyl ether, mixed on a Vortex for 30 s and centrifuged at 1800 *g* for 5 min. The organic layer was transferred to clean tubes and evaporated to dryness using a Savant Speed Vac concentrator–evaporator.

The above extraction procedure was also applied to urine samples. The exception, however, was that all urine samples were diluted 100-fold, with water, prior to extraction.

The residues remaining after evaporation were derivatized with a 0.1% (v/v) solution of NEIC in chloroform (0.20 ml) and mixed for 30 s. Aliquots of 0.015 to 0.20 ml were injected into the HPLC system.

Extraction yields

A chloroform solution of AC (solution 2) was evaporated to dryness. These samples ($n = 4$) were then reconstituted to give final concentrations of 25 and 500 ng/ml and 2 and 100 mg/l in plasma and urine, respectively. The solutions were extracted in the absence of I.S., and exactly 4 ml diethyl ether were taken off. The organic layer was evaporated, and the samples were derivatized as described above. The peak areas of AC extracted versus unextracted equivalent concentrations of drug were compared under identical chromatographic conditions.

Derivatization yields

Acebutolol (solution 2) was added to dry test tubes ($n = 4$) to give concentrations of 50 and 500 ng/ml in 0.01% (v/v) triethylamine–chloroform. The samples were evaporated to dryness and derivatized as described above. The amount of underivatized AC was measured. To determine the unreacted AC, the mobile phase was modified to isopropyl alcohol–chloroform–triethylamine (80:20:0.01, v/v), and the fluorescence detector set to 238 and 389 nm for excitation and emission, respectively. The derivatization yields were calculated by comparing peak areas of AC to those of derivatized AC.

Accuracy and precision

To determine accuracy, AC was added to plasma ($n = 9$) and concentrations of individual enantiomers were calculated using a standard curve. The difference between the mean estimated and the mean added concentrations was taken as the accuracy of the method. Precision was estimated by determining the inter-assay coefficient of variation (C.V.).

Applicability to other compounds

Compounds possessing a secondary amine with a hydroxy group in the β-position were examined. This included atenolol, alprenolol, bupranolol, labetolol, metoprolol, nadolol, pindolol, propranolol, sotalol, toliprolol and tocainide.

Treatment of data

Concentrations of each enantiomer of AC were determined by the ratio of AC/I.S. peak areas. In each case, the first I.S. peak to elute was used in these calculations. All results are reported as mean ± S.D.

RESULTS AND DISCUSSION

Chiral derivatization with subsequent separation of diastereoisomers has recently been reported for a number of β-adrenoceptor blocking drugs [2,3,7,8]. Either S-($-$)- or R-($+$)-1-PEIC has been used for separation and determi-

nation of some of these compounds [2,3,8]. Without exception, the resultant diastereoisomers have been the urea, as opposed to the carbamate derivatives.

The only reports of separation of AC, to date, utilize either TPC [10] or PEIC [18] as derivatizing reagents. The shortcomings of TPC have been documented [2,18]. Previous studies with PEIC suggest that a somewhat lengthy reaction time is required [3]. Moreover, after clinical doses of AC in patients, these previously reported methods lacked sensitivity, despite the use of fluorescence detection [18]. We decided to use NEIC as an alternative to PEIC as fluorescence and, hence, sensitivity of the derivative might be augmented. Other investigators have suggested NEIC for derivatization of enantiomers where increased sensitivity is necessary [5,19].

Our attempts to separate enantiomers of AC using reversed-phase HPLC, although successful, resulted in an additional peak eluting as late as 90 min after injection. The identity of this peak, however, is unknown but may be a dimer or trimer of NEIC. This limited the convenience of the assay under isocratic conditions. Moreover, if a large excess of NEIC was used, or if derivatization was conducted under aqueous conditions, this peak became more abundant. The peak, however, could be diminished in size by (1) the addition of ethanolamine, as suggested by Pflugmann et al. for metoprolol and PEIC [3] to stop the reaction or (2) by utilizing normal-phase HPLC. The first alternative was not feasible, as it did not entirely eliminate the peak. We, therefore, chose normal-phase HPLC as the better alternative, as this peak was absent under these conditions.

With normal-phase HPLC, virtual baseline separation of the peaks corresponding to AC was attained (resolution, $R_s = 1.45$). AC enantiomers eluted at about 12 and 13 min (R-AC and S-AC, respectively); pindolol enantiomers (I.S.) eluted at approximately 6 and 7 min. The exact order of elution of the peaks was determined using pure S-AC and R-AC. Fig. 1 depicts representative chromatograms of drug-free plasma, a spiked plasma sample (10 ng/ml) and plasma taken from a normal volunteer 12 h after a 200-mg oral racemic dose. Fig. 2 depicts drug-free urine and a urine sample taken from a 12–24 h collection interval in the same volunteer.

The extraction yields of AC in plasma were 91.1 ± 7.8 and $80.4 \pm 7.0\%$ for 25 and 500 ng/ml, respectively. In urine, extraction yields were 80.8 ± 3.85 and $87.4 \pm 7.19\%$ for 2 and 100 mg/l, respectively.

The derivatization yields were found to be 100% within 10 min, as no underivatized drug could be detected. These observations agree with others, indicating that similar reactions utilizing isocyanates as derivatizing reagents are rapid [8,18]. Contrary to these findings with AC, a previous study [5] which utilized NEIC for separation and determination of betaxolol enantiomers found that incubation of samples at 37°C for 1.5 h was necessary.

In all cases, excellent linearity was observed between the peak-area ratios of S-AC/I.S. and R-AC/I.S. in both plasma and urine ($r > 0.998$). Typical plasma
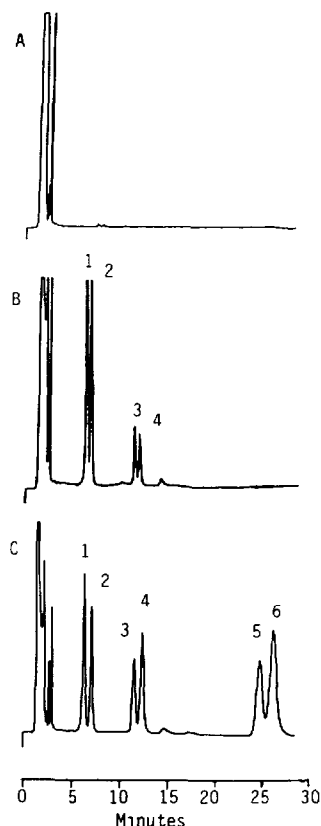
Fig. 1. Chromatograms of (A) drug-free plasma, (B) plasma spiked with 10 ng/ml of each enantiomer of AC and (C) a plasma sample taken 12 h following oral administration of a single 200-mg racemic dose of AC to a healthy subject. Peaks: 1 and 2 = pindolol diastereoisomers; 3 and 4 = AC diastereoisomers; 5 and 6 = diacetolol diastereoisomers (identification not confirmed). Full-scale recorded output range = 0.1 μA

calibration curves could be described by $y = 0.075 + 0.071x$ and $y = 0.067 + 0.066x$ for the R- and S-enantiomers, respectively. In urine, typical curves were $y = -0.092 + 0.119x$ and $y = -0.078 + 0.111x$ for R-AC and S-AC, respectively, y is the peak-area ratio (S- or R-AC/I.S.) and x is the enantiomer concentration.

The observed intra-assay C.V. was always less than 10% over the examined concentration range in plasma (Table I). Although the lowest concentration examined in plasma was 10 ng/ml, the actual sensitivity is 1 ng/ml if one uses a signal-to-noise ratio of 4:1.

Identification of the two peaks corresponding to AC enantiomers indicated that the urea, and not the carbamate, derivative was formed. The molecular ion (MH^+) was observed at m/z 534. Another ion at m/z 516 suggests the loss of H_2O. These results indicate that the derivative is mono-substituted. Fur-
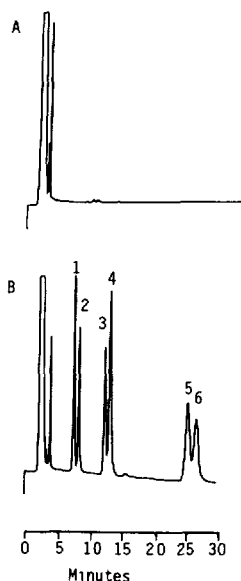
Fig. 2 Chromatograms of (A) drug-free urine and (B) urine collected from a healthy subject 12–24 h following a single oral dose of 200 mg racemic AC. For peak identification, see Fig. 1. Full-scale recorder output range $-0.1\ \mu$A.

TABLE I

ACCURACY AND PRECISION OF THE METHOD

$n=9$ (three sets for three days).

Concentration added (ng/ml)	Concentration measured (mean ± S.D.) (ng/ml)		Accuracy (error, %)		Precision (C V., %)	
	R	S	R	S	R	S
10	10.4 ± 0.46	10.4 ± 0.42	3.5	4.4	4.5	4.0
25	25.3 ± 1.26	25.0 ± 1.00	1.1	0.1	4.9	4.0
50	51.9 ± 4.16	51 4 ± 3.53	3.8	2.8	8.0	6.9
100	101 ± 7.25	100 ± 6.06	1.7	0.3	7.1	6.0
200	204 ± 15.9	202 ± 16.0	2.1	1.4	7.8	7.9
500	499 ± 3.03	499 ± 3.36	−0.1	−0.1	0.70	0.67

thermore, substitution must be on the nitrogen atom, as the loss of H_2O suggests a free hydroxyl group. Consistent with other reports [2,18], it was noted that NEIC in large excess did not result in formation of the carbamate derivative. Similarly, increasing the reaction time of derivatization did not result in formation of the carbamate derivative. Based on this finding, it may be rea-
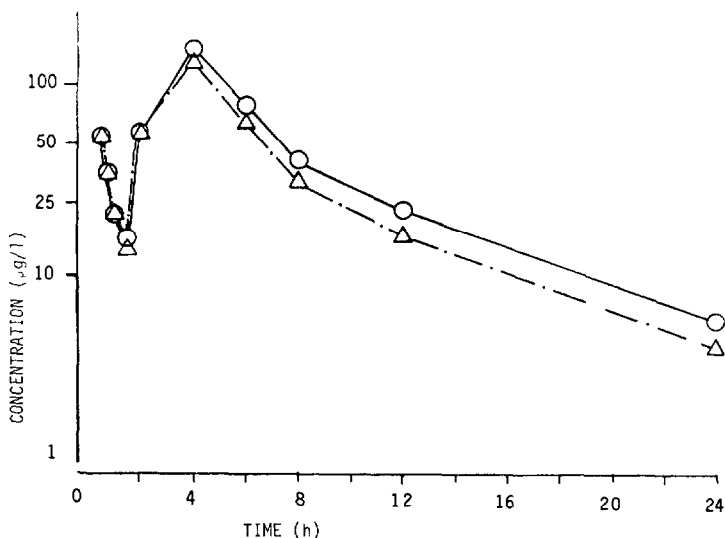
Fig. 3 Plasma concentration versus time profile of S-AC (○) and R-AC (△) in a healthy volunteer following a single oral 200-mg racemic dose of AC.

sonable to suggest that similar compounds possessing the secondary amino group, β to the hydroxyl functional group, form urea derivatives.

As we did not have access to authentic metabolite (diacetolol) during the course of this work, we were unable to identify and quantify peaks which might correspond to diacetolol enantiomers. We did, however, observe that two peaks elute at 25 and 27 min and these could be the derivatized diacetolol enantiomers. Investigation of these peaks is of future concern.

The plasma versus time course of 200 mg AC given orally to a healthy female volunteer is shown in Fig. 3. Although a previous report [10] suggests that the disposition of AC is not stereoselective, these findings suggest a trend of stereoselectivity in this patient; S-AC concentrations are consistently greater than those of R-AC. This discrepancy may be due to the fact that the previous study utilized TPC as a derivatizing reagent. Racemization, as well as contamination (up to 15%) with the (+)-enantiomer of the TPC [18] may explain some of these discrepancies.

In conclusion, the assay described is rapid and sensitive, allowing for numerous samples to be processed in a short period of time. Perhaps more importantly, this method appears to be a 'general' method for the determination of a number of compounds which possess a secondary amino moiety β to the hydroxy group. This derivatization method appears to have wide applicability as atenolol, nadolol, pindolol, propranolol, sotalol, toliprolol and tocainide were successfully separated and detected. Only minor modifications to the mobile

phase, excitation and emission wavelengths were required for detection of these compounds. Further studies of these compounds are being conducted.

ACKNOWLEDGEMENTS

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