Research Article

Biomedical Chromatography

Received: 21 October 2008, Accepted: 11 November 2008 Published online 24 March 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/bmc.1187

Reversed-phase liquid chromatographic determination of enantiomers of atenolol in rat plasma using derivatization with Marfey's reagent

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ABSTRACT: An HPLC method was established for enantioseparation of (*R*,*S*)-atenolol (ATE) and determination of enantiomers in rat plasma. Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDNP-L-Ala-NH_2, MR) was used as chiral derivatizing reagent with detection of diastereomers at 340 nm. It was shown that the *R*-isomer eluted before the *S*-isomer. The method was validated for linearity, repeatability, limits of detection and limit of quantification (LOQ). Recovery of ATE at LOQ was 92.8% for (*R*)-ATE and 92.6% for (*S*)-ATE. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: (*R*,*S*)-atenolol; rat plasma; Marfey's reagent; diastereomers; RP-HPLC

Introduction

Atenolol belongs to commonly known group of β-blockers and is used to treat hypertension, coronary heart disease, arrhythmias, sinus tachycardia and myocardial infarction, where it acts preferentially upon the β-adrenergic receptors in the heart (Gilman and Goodman, 1985). It is a chiral hydroxyl amine-containing compound, 2-{*p*-[2-hydroxy-3-(isopropylamino) propoxy] phenyl} acetamide. Although marketed as a racemic mixture, the (*S*)-enantiomer possesses much greater affinity for binding to the β-adrenergic receptors than the *R*-antipode (Mehvar and Brocks, 2001). The two enantiomers should be considered as different drugs and a clear picture of their pharmacodynamic and pharmacokinetic profiles is likely to emerge only when the fate of each enantiomer is established. Significant differences in the pharmacokinetics of ATE enantiomers were found in humans and rats (Boyd *et al*., 1989; Enquist and Hermansson, 1989; Mehvar *et al*., 1990). The direction and extent of these differences are the same in humans and rats (Mehvar *et al*., 1990).

Some of the reports on analysis/determination of ATE in biological samples include chiral assay in microdialysis and plasma samples of rats using chiral cellobiohydrolase as stationary phase (Fornstedt *et al*., 1997), separation and quantitation of (*R*)- and (*S*)-ATE in human plasma and urine using an α1-AGP column (Enquist and Hermansson, 1989), direct enantiospecific HPLC bioanalysis of (*R*,*S*)-ATE using chiral stationary phase consisting of cellulose tris-3,5-dimethylphenylcarbamate, coated on silica gel; Kofahl *et al*., 1993) and stereoselective HPLC bioanalysis of (*R*,*S*)-ATE in the form of oxazolidine-2-one derivatives in plasma with a comparative human pharmacokinetic study using (*R*,*R*)-diaminocyclohexane-dinitrobenzoyl chiral stationary phase (Egginger *et al*., 1993). RP-HPLC analysis of ATE enantiomers in plasma has also been reported after chiral derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate (Flec) (Rosseel *et al*., 1991).

Agbaba and Ivković (2007) have provided a comprehensive report on TLC chiral separation of different β-blockers. Direct enantiomeric resolution of racemic ATE, propranolol and metoprolol has been reported by Bhushan *et al*. by TLC using certain chiral selectors as impregnating reagents; these include L-lysine and L-arginine (Bhushan and Thiongo, 1998), L-aspartic acid (Bhushan and Arora, 2003), Cu(II)-L-arginine complex (Bhushan and Gupta, 2006) and L-tartaric acid, (*R*)-mandelic acid and (–)-erythromycin (Bhushan and Tanwar, 2008).

Indirect separation of pairs of diastereomers is sometimes simpler to perform, and may produce better results than direct separation because chromatographic conditions can be optimized more easily. Marfey introduced 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDNP-L-Ala-NH_2, FDAA; Marfey's reagent MR) and used it for preparation of diastereomeric amino acids followed by their reversed-phase HPLC separation (Marfey, 1984). The amide is chosen because it is quite stable, neutral and apparently not easily racemized. There are two review articles which extensively cover the characteristics, applications, advantages and limitations of this reagent and its chiral variants (B'Hymer *et al*., 2003; Bhushan and Brückner, 2004).

Keeping in view the presence of amino group in ATE, the characteristics and literature reports on MR, our continued work on enantiomeric resolution of β-blockers including atenolol, as noted above, and in search of new chiral reagents for its enantiomeric resolution we report in this paper resolution and determination of enantiomers of ATE in plasma of rats. For this purpose diastereomers of (*R*,*S*)-ATE were prepared with

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Abbreviations used: ATE, (*R*,*S*)-atenolol; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; MR, Marfey's reagent; TFA, trifluoroacetic acid.

FDNP-L-Ala-NH_2 (the MR), which were then separated by RP-HPLC. The separation method was validated for linearity, accuracy, limit of detection and limit of quantification. To the best of authors' knowledge there is no earlier report on chiral HPLC assay of ATE enantiomers in rat plasma using Marfey's reagent.

Experimental

Materials and Apparatus

(*R*,*S*)- and (*S*)-ATE, 1,5-difluoro-2,4-dinitrobenzene (DFDNB) and L-Ala-NH_2 were obtained from Sigma-Aldrich (St Louis, MO, USA). Trifluoroacetic acid (TFA) and other chemicals of analytical grade were purchased from S.D. Fine Chemicals (Mumbai, India).

The HPLC system (Knauer, Berlin, Germany) consisted of a pump1000 (with a 10 mL head), manager/degasser 5000, UV detector 2500, manual injection valve and Eurochrom operating software. The C_{18} column (250 × 4.6 mm i.d., 5 μm) was from Knauer. Both Centrifuge C-24 BL and Incubator CI-65 were from Remi, Mumbai, India. The Milli-Q system of Millipore (Bedford, MA, USA) was used to purify double-distilled water to HPLC-grade deionized water.

Preparation of Solutions and Chromatography

Stock solution of (*R*,*S*)- and (*S*)-ATE (50 mM) was prepared in 0.1 M $NaHCO_3$. Reagent solutions (1%) were prepared in acetone and stored in refrigerator at 0—4°C. Solutions of $NaHCO_3$ (0.1 M), HCl (2 M) and 0.01 M TFA were prepared in purified water. All solutions and samples were filtered through a 0.45 μm filter.

Acetonitrile–0.01M–aqueous TFA was used as mobile phase under a linear gradient of acetonitrile from 35 to 65% in 45 min. The flow rate was 1.0 mL/min with UV detection at 340 nm. The load amount of 20 μL was injected onto the column. Mobile phase for HPLC was filtered through a 0.45 μm filter and degassed before use.

Method Validation

Selectivity. The chromatograms of blank, spiked and drug administered rat plasma samples were compared to determine the interference by endogenous co-eluent components in the enantiomeric separation of ATE.

Limit of quantification. Batches of blank plasma spiked with different concentrations of ATE ($n = 5$) were analyzed to determine limit of quantitation (LOQ). The LOQ was defined as the lowest concentration of spiked sample with recovery within the range 82–103% and relative standard deviation (RSD) less than 2%.

Calibration curve. The calibration curve was generated by linear regression of the peak area (y) against the concentration (x) of ATE in rat plasma. The acceptance criteria for calibration curve was a correlation coefficient (r^2) of >0.99.

Animal Experiments

Male Wistar (*Rattus norvegicus*) rats weighing 200–250 g (6 weeks old) were purchased from National Institute of Pharmaceutical Education and Research (Mohali, India), and were housed in an environmentally controlled room with a 12 h light–dark cycle. Physiological saline (0.9% NaCl) was used to prepare a solution of (R,S)-ATE (4 mg/mL); it was administered intravenously to the conscious animals at a dose of 10 mg/kg body weight. Similarly, (S)-ATE was also administered to another rat. Blood samples (500 μL) were withdrawn at 1, 3, 6, 12, 24 and 28 h, after drug administration, and collected in heparinized tubes. The blood samples were immediately centrifuged at 5000 rpm for 5 min (4°C) and the plasma obtained was stored at −20°C until analysis. To each of the plasma sample (about 250 μL), water (100 μL) and $NaHCO_3$ solution (50 μL, 1 M) were added.

Derivatization with MR

Plasma sample (50 μL) containing (*R*,*S*)-ATE was added to the solution of FDNP-L-Ala-NH_2 (100 μL of 10 mM in acetone), in a Teflon tube. The reaction mixture was kept at 45°C in an incubator for 90 minutes. After cooling to room temperature, HCl (2 M, 10 μL) was added to terminate the reaction followed by addition of acetonitrile (200 μL) to dissolve the diastereomeric derivatives. The resulting solution was degassed and filtered and the diastereomers were analysed by RP-HPLC. Derivatization of plasma samples containing (*S*)-ATE was carried out in the similar manner.

Results and Discussion

MR was synthesized using DFDNB and L-Ala-NH2 as per procedures optimized and reported in literature (Marfey, 1984; Brückner and Keller-Hoehl, 1990; Bhushan and Brückner, 2004) and characterized (Bhushan and Kumar, 2008). The basic approach reported in the literature (Bhushan *et al*., 2008) for derivatization of amino acids was adopted for derivatization of (*R*,*S*) and (*S*)-ATE in the plasma samples. Plasma samples were used for derivatization reaction, as described above, after extraction with *n*-hexane. Extraction with other solvents like *n*-hexane–*n*-butanol (50:50, v/v) and *n*-butanol–dichloromethane (30:70 v/v) resulted in recoveries of less than 55% and therefore was not used.

MR reacts stoichiometrically, without racemization, with primary and secondary amino groups. Diastereomeric derivatives prepared with MR have strong absorbance at 340 nm due to the presence of highly absorbing dinitrophenyl chromophore, which makes them suitable for chromatographic applications where highly sensitive detection and quantification are required. Since the diastereomers are light-sensitive, the reaction at every step was protected from light and the derivatives were kept in dark at 0–4°C.

RP-HPLC

The resolution was achieved using linear gradient of acetonitrile (from 35 to 65%) and 0.01 M TFA in 45 min. The peaks of diastereomers of ATE, prepared with MR, are clearly observed in the chromatogram of plasma spiked with 100 nM ATE [Fig. 1(B)], and there were no interfering peaks from endogenous substances in the blank chromatogram [Fig. 1(A)]. The peaks were successfully detected in the chromatogram of rat plasma at 3 h after administration [Fig. 1(C)]. Figure 2 shows the time-course profile of the enantiomers of ATE in rat plasma after intravenous administration of ATE at a dose of 10 mg/kg body weight ($n = 3$). It was observed that the concentration of ATE decreased gradually after 3 h, and at 28 h of administration the concentrations of (*R*)- and (*S*)-isomers were 10.5 and 8 ng/mL, respectively. The (*R*)-isomer was found to elute before the (*S*)-isomer. The concentration of (*R*)-ATE was found to be higher than that of (*S*)-ATE at each time of measurement though the difference between the two was small (Fig. 2).

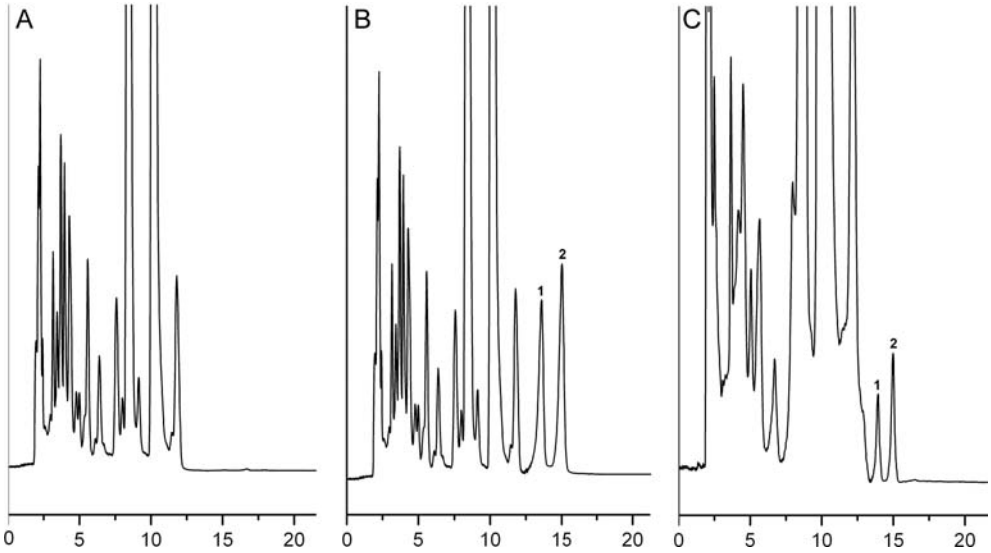

Figure 1. Representative chromatogram of derivatized ATE in plasma extract; peaks 1 and 2 are (*R*)-ATE and (*S*)-ATE, respectively. (A) Blank plasma; (B) plasma spiked with ATE (100 nM); (C) plasma 3 h after intravenous administration of ATE (10 mg/kg).

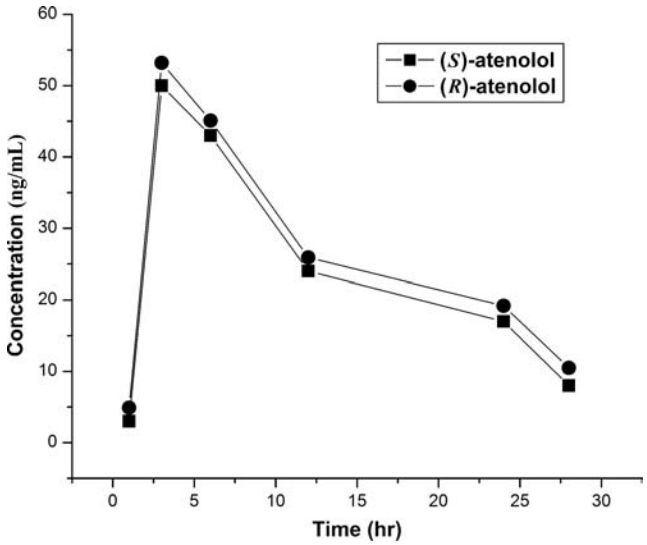

Figure 2. Time course for the concentration of ATE enantiomer in rat plasma after intravenous administration of ATE (10 mg/kg, $n = 3$).

Since (*S*)-ATE was also administered to a separate set of rats, the analysis of their plasma samples (prepared from blood samples withdrawn at different time intervals of 3, 6, 12, 24 and 28 h) via diastereomerization with MR followed by HPLC (under the same chromatographic conditions) was carried out. None of them showed the presence of (*R*)-ATE. It can be concluded that no biotransformation or racemization of (*S*)-ATE to (*R*)-ATE was occurring. It was observed that (*S*)-ATE remained in its original form.

Selectivity

Under the assay conditions, enantiomers of atenolol were completely separated, and no interference was observed in the blank plasma. The retention time was 14.2 and 15.6 min for (*R*)-ATE and (*S*)-ATE, respectively. The results provide a specific and selective HPLC method for the determination of ATE in rat plasma after intravenous administration. The coefficients of variation (CV %) of retention times of (*R*)-ATE and (*S*)-ATE were less than 0.5% ($n = 5$) within the series of runs (intra-day) and less than 1% ($n = 5$) between the inter-day series of runs. The retention time range was 14.1–14.5 min [for (*R*)-ATE, $n = 5$], and 15.4–15.8 min [for (*S*)-ATE, $n = 5$], on a day-to-day basis.

Limit of Quantification

LOQ for either of the enantiomer of ATE by this method was 3 ng/mL, which was the lowest concentration on the calibration curve with precision of 2.5% and accuracy of 92.8%.

Stability

Experiments with three freeze–thaw cycles of the samples at −20°C for low and high concentrations for 15 days showed that ATE was stable in rat plasma under this condition and there was found no significant decrease of (*R*)- and (*S*)-ATE. The stability test of the processed samples indicated that ATE was stable at room temperature for 24 h, allowing sufficient time for sample preparation and HPLC analysis.

Linearity

The calibration curves were linear within the range from 3 to 300 ng/mL for both (*R*) and (*S*)-ATE. The linear equation was $y = 11.18x - 0.12$ ($r^2 = 0.999$) for (*R*)-ATE and $y = 10.3x - 0.5$ ($r^2 = 0.998$) for (*S*)-ATE.

Accuracy and Precision

The accuracy and precision was assessed by analysis of samples at low and high concentration levels. The recovery of ATE enantiomers from plasma was evaluated by comparing the measured concentration of five replicate samples with the corresponding spiked concentration at LOQ, and at low and high levels of 0.098

Table 1. Within-day and day-to-day reproducibility for the quantification of (*R*)- and (*S*)-ATE in rat plasma ($n = 5$)

Theoretical concentration (μg/mL)	Measured concentration (μg/mL)							
	(*R*)-Atenolol				(*S*)-Atenolol			
	Mean	±SD	CV (%)	Recovery (%)	Mean	±SD	CV (%)	Recovery (%)
	Intra-day variations (five replicates at each concentration)							
0.098	0.097	0.001	1.03	98.9	0.099	0.001	1.01	101.0
0.187	0.185	0.001	0.54	98.9	0.185	0.001	0.54	98.93
	Inter-day variations (five replicates at each concentration)							
0.098	0.096	0.001	1.04	97.9	0.092	0.003	3.26	93.8
0.187	0.194	0.003	1.54	103.7	0.191	0.002	1.04	102.0

CV, coefficient of variation; SD, standard deviation.

and 0.187 μg/mL. The recoveries at low and high concentration are shown in Table 1. Recovery at LOQ was 92.8% for (*R*)-ATE and 92.6% for (*S*)-ATE.

The precision was evaluated by intra- and inter-day variability in terms of RSD. Samples were consequently analysed in a single day for intra-day precision, and was repeated for five consecutive days for inter-day precision. Inter- and intra day precisions were assessed by calculating the relative standard deviation of the control sample concentration measured in each validation run. Intra-day and inter-day precisions were investigated by five-fold assay of plasma samples spiked with 0.098 and 0.187 μg/mL of each of ATE enantiomers. Intra-day assay accuracy and precision ranged from 98.9 to 101% and from 0.51 to 0.54%, respectively. Inter-day assay accuracy and precision ranged from 93.8 to 103.7 and from 1.04 to 3.26 respectively, as shown in Table 1.

Conclusion

The method reported here is highly sensitive and selective for determination of ATE enantiomers in rat plasma. It offers the advantage of being simple and convenient for clinical pharmacological studies of ATE enantiomers. The method was applied to measure concentration of ATE enantiomers in plasma after intravenous administration of racemic atenolol to rats. Enantiomers of (*R*,*S*)-ATE were well resolved on an achiral C_{18} column after derivatization with Marfey's reagent. The method is more sensitive in comparison to the literature report (Rosseel *et al*., 1991), where 100 μL plasma was used and the quantitation limit was 10 ng/mL, while in the present case only 50 μL of plasma was used and the LOQ was 3 ng/mL for each enantiomer of atenolol.

Acknowledgements

The authors are grateful to University Grants Commission (UGC) India, New Delhi, for the award of junior research fellowship to (S.T.). The authors thank Professor S.M. Sondhi (Chemistry Department) and Dr P. Roy and Ms A. Chakraborty (Department of Biotechnology, IIT Roorkee) for their technical assistance. Thanks are also due to AvH-Stiftung, Bonn, Germany for kindly donating Knauer HPLC system (to R.B.).

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