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Enantiomeric separation of metoprolol and α-hydroxymetoprolol by liquid chromatography and fluorescence detection using a chiral stationary phase

Bipin Mistry, James L. Leslie, Natalie D. Eddington*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 100 Penn Street, AHB, Baltimore, MD 21201-6808, USA

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Abstract

A sensitive and stereoselective high-performance liquid chromatographic assay for the determination of the enantiomers of metoprolol (*R*- and *S*-) and the diastereoisomers of α-hydroxymetoprolol (IIA, IIB) in plasma is reported. Chromatography involved direct separation of enantiomers using a Chirobiotic T bonded phase column (250×4.6 mm) and a mobile phase consisting of acetonitrile–methanol–methylene chloride–glacial acetic acid–triethylamine (56:30:14:2:2, v/v). Solid-phase extraction using silica bonded with ethyl group (C_2) was used to extract the compounds of interest from plasma and atenolol was used as the internal standard. The column effluent was monitored using fluorescence detection with excitation and emission wavelengths of 225 and 310 nm, respectively. *S*-Metoprolol, *R*-metoprolol, IIB and IIA eluted at about 5.9, 6.7, 7.3 and 8.2 min without any interfering peaks. The calibration curve was linear over the range of 0.5 to 100 ng/ml for each isomer of metoprolol and 1 to 100 ng/ml for each isomer of α-hydroxymetoprolol (IIA & IIB). The mean intra-run accuracies were in the range of 96.2 to 114% for *R*-metoprolol, 94.0 to 111% for *S*-metoprolol, 90.2 to 110% for IIA, and 94.6 to 106% for IIB. The mean intra-run precisions were all in the range of 2.2 to 12.0% for *R*-metoprolol, 2.1 to 11.1% for *S*-metoprolol, 1.9 to 14.5% for IIA, and 3.2 to 11.0% for IIB. The lowest level of quantitation for the enantiomers of metoprolol was 0.5 ng/ml and 1.0 ng/ml for α-hydroxymetoprolol (IIA and IIB). The absolute recoveries for each analyte was ≥95%. The validated method accurately quantitated the enantiomers of parent drug and metabolite after a single dose of an extended release metoprolol formulation.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Metoprolol; α-Hydroxymetoprolol

1. Introduction

Metoprolol [(I); {1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-2-propanol}] is a cardioselective β-adrenergic blocking agent and like other β-blockers it has an asymmetric center in its amino alcohol side chain [1–3]. It is commercially available as the tartrate and used as a racemic mixture in dosage forms [*R*-metoprolol/*S*-metoprolol: 50/50]. Its structures and metabolic pathways are shown in Fig. 1. These hepatic pathways are known to be stereoselective and polymorphic [3]. The majority of the dose (85–95%) is metabolized via *O*-demethylation and benzylic oxidation [1–3]. These pathways

*Corresponding author. Tel.: +1-410-7066-710; fax: +1-410-7066-580.

E-mail address: neddingt@rx.umaryland.edu (N.D. Eddington).

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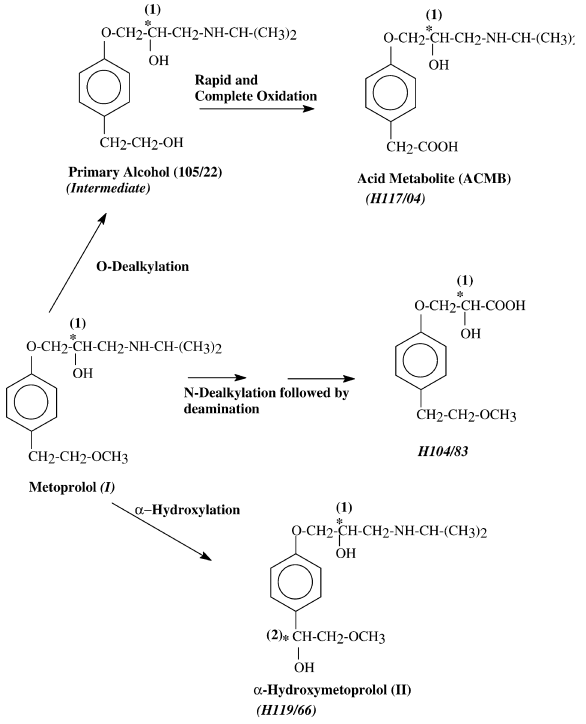

Fig. 1. Major pathways of first-pass metabolism of metoprolol. The (*) represents the chiral carbon and the numbers (1) and (2) denote chiral center numbers.

are polymorphic in nature. The benzylic oxidation product, α-hydroxymetoprolol (II), is a diastereomer due to the generation of an additional chiral center. The diastereomers and stereoisomers of α-hydroxymetoprolol are 1*S*2*S*, 1*S*2*R*, 1*R*2*R* and 1*R*2*S* and (1*S*2*S*, 1*S*2*R*)=IIB and (1*R*2*R*, 1*R*2*S*)=IIA have been chromatographically separated in this laboratory (Fig. 1). These pairs represent the formation of IIA from *R*-metoprolol (*R*-I) and the formation of IIB from *S*-metoprolol (*S*-I) after α-hydroxylation. In a similar manner the *R* and *S* isomers of acid metabolite (*R*-ACMB and *S*-ACMB) are formed metabolically from *R*- and *S*-metoprolol, respectively

Most chromatographic enantiomeric separations are classified in two general categories, indirect and direct method of separation. Both methods have been applied to the separation of metoprolol isomers [4–19] and similar classes of other β-blockers. Indirect methods [4–12] require derivatization of the asymmetric molecule with an optically pure derivatizing agent to form diastereomeric pairs which can be resolved using chromatographic methods such as high-performance liquid chromatography (HPLC) or gas–liquid chromatography (GLC) with conventional stationary phases (e.g., C_8, C_{18}). Direct methods [13–19] involve the use of specialty chiral stationary phases or mobile phase additives. Improved resolution between isomers can be achieved by achiral derivatization of analyte isomers along with chromatography using chiral stationary phases.

Recently, chiral chromatography methods have been developed for the enantiomers of metoprolol in plasma and urine [20,21]. These methods use either solid-phase extraction (SPE) or liquid–liquid extraction techniques and sensitivity ranges from 5 to 25 ng/ml. However, neither method quantifies the hydroxymetoprolol enantiomers. A stereoselective assay for metoprolol (*R*-I and *S*-I) and its metabolites (IIA, IIB, *R*-ACMB and *S*-ACMB) was needed to evaluate the concentration of metoprolol and metabolite isomers in human plasma after administration of extended release formulations. The objective of this work was to develop and validate a sensitive, rapid, stereospecific and simultaneous method for α-hydroxymetoprolol isomers (IIA and IIB) and metoprolol isomers in a large number of human plasma samples. The stereospecific analysis for the acid metabolite required a different chromatographic conditions and is reported elsewhere [22].

2. Materials and methods

Metoprolol tartrate and atenolol were obtained from Sigma (St. Louis, MO, USA). The α-hydroxymetoprolol benzoate salt was a gift from Ciba-Geigy and α-hydroxymetoprolol base was supplied by Dr. Jeremy Wright (Department of Pharmaceutical Sciences, University of Maryland, Baltimore, MD, USA). *R*-Metoprolol and *S*-metoprolol were gifts from Astra (Sweden). Methyl 4-hydroxy phenylacetate, epichlorohydrin and isopropylamine were obtained from Aldrich. Sodium hydroxide, 1 *M* hydrochloric acid (HCl), glacial acetic acid, chloroform, toluene, acetonitrile, methanol, hexane, 1,2-dichloroethane and methylene chloride were of HPLC grade or at least certified ACS quality (Fisher, PA, USA). Deionized water was prepared by an ultrapure water

system Picotech II Plus[R], Hydro (Research Triangle Park, NC, USA).

C_2 SPE columns, 1 ml, were obtained from Varian (Harbor City, CA, USA) and C_{18} SPE columns, 1 ml, were obtained from Waters (Milford, MA, USA). A chiral separation kit containing five different Pirkle type (50×3.2 mm) columns was obtained from Phenomenex (Torrance, CA, USA). The chiral phases were: Chirex (*S*)-*tert*.-leucine & (*R*)-1-α-naphthylethylamine (3020), Chirex (*S*)-indoline carboxylic acid & (*R*)-NEA (3022), Chirex (*S*)-valine & (*R*)-1-α-naphthylethylamine (3014). A Zorbax RX C_8 (250×4.6 mm) analytical column was obtained from McMod (PA, USA) and a C_4 (150×4.6 mm) analytical column obtained from MetaChem Technology (Torrance, CA, USA). Columns with β-cyclodextrin (Cyclobond I), α-cyclodextrin (Cyclobond III), γ-cyclodextrin (Cyclobond II) and macrocyclic antibiotic bonded phases (Chirobiotic T (Teicoplanin) and Chirobiotic V (Vancomycin), 250×4.6 mm, were obtained from Advance Separation Technologies (Whippany, NJ, USA).

2.1. Chromatographic resolution involving direct methods of separation

Several direct methods of separation of metoprolol and metabolite enantiomers were evaluated. A chiral separation kit containing several Pirkle type columns (50×4.6 mm) was investigated for this purpose. These phases offers a chiral environment, π–π interactions and chiral hydrogen bonding sites. All Pirkle type columns, which were urea-type bonded phases, were tried using the recommended mobile phase combinations for separation of the stereoisomers of β-blockers (Phenomenex). The mobile phases were combinations of trifluoroacetic acid, methanol or ethanol, hexane, dichloroethane, chloroform and methylene chloride at a low flow-rate (0.2 ml/min). The injected analytes were dissolved in mobile phase. Only one column, No. 3022, which had previously been shown to be capable of resolving all four diasteriomers of labetolol, was able to resolve metoprolol enantiomers with retention times of 12.4 and 13.1 min and α-hydroxymetoprolol isomers as broad peaks with retention times of 25.5 and 28.2 min. There was no success in separating all four diastereomers of α-hydroxymetoprolol using Pirkle type stationary phases.

A HPLC system equipped with column switching station capable of handling eight different columns was employed to facilitate the column and mobile phase selection. Columns which can separate analytes by inclusion complex formation such as native cyclodextrin (α, β and γ), derivatized cyclodextrin bonded phases and antibiotics (teicoplanin and vancomycin) bonded phases to silica gel were investigated.

2.2. Stereospecific HPLC methods

2.2.1. Reagents and standard preparations

2.2.1.1. R-Metoprolol/S-metoprolol/IIA/IIB plasma standards. A 1-ml volume of the metoprolol and 4.0 ml of α-hydroxymetoprolol stock solutions were combined in a 100-ml volumetric flask and diluted to the volume with deionized water to prepare 10 000 ng/ml each of *R*-metoprolol/*S*-metoprolol/IIA/IIB stock dilution in water. A 2.0-ml portion of this stock dilution was further diluted with blank human heparinized plasma (Biological Specialty, Colmar, PA, USA) matrix in a 100-ml volumetric flask to prepare 100/100/100/100 ng/ml *R*-metoprolol/*S*-metoprolol/IIA/IIB standard. These solutions were used subsequently to prepare plasma standards with concentrations of 100/100/100/100, 50/50/50/50, 25/25/25/25, 15/15/15/15, 7.5/7.5/7.5/7.5, 4/4/4/4, 2/2/2/2, 1/1/1/1 and 0.5/0.5/–/– ng/ml of *R*-metoprolol/*S*-metoprolol/IIA/IIB. Also, control samples in plasma were prepared at concentrations 50/50/50/50, 15/15/15/15, 7.5/7.5/7.5/7.5, 2/2/2/2, 1/1/1/1 and 0.5/0.5/–/– ng/ml of *R*-metoprolol/*S*-metoprolol/IIA/IIB. Five replicates of these control samples were analyzed with each validation run.

Control samples in plasma at concentrations 50/50/50/50, 15/15/15/15, and 7.5/7.5/7.5/7.5 were analyzed with subject samples. One set of control samples was inserted into the run after approximately every 17 subject samples. The exact concentrations of the standards used in validations and clinical studies were calculated based on the actual concentration of the stock solutions.

Control samples in plasma at concentrations 1000/

1000, 500/500 and 100/100 were analyzed with subject samples. One set of control samples was inserted into the run after approximately every 17 subject samples. The exact concentrations of the standards used in the validations and clinical studies were calculated based on the actual concentrations of the stock solutions.

2.2.2. Sample preparation

SPE using silica bonded with ethyl group (C_2) was suitable for extracting α-hydroxymetoprolol and metoprolol from plasma [23]. The β-blocker, atenolol, was found to be a suitable internal standard for this assay. C_2 SPE columns were placed into a Vac Elut (Varian, Harbor City, CA, USA) vacuum manifold and each column was conditioned with 2 ml of acetonitrile (ACN) followed by 2 ml of water. One ml of plasma standard, sample or control sample was drawn through each column followed by 0.2 ml of internal standard solution (1000 ng/ml atenolol). Each column was washed with three volumes of deionized water and then by three volumes of ACN–water (50:50, v/v). The analytes and internal standard were then eluted with two 0.2 ml portions of 0.1 *M* HCl–ACN (50:50, v/v) and the eluent evaporated to dryness at 45°C in a water bath with a gentle flow of nitrogen (25–30 p.s.i.; 1 p.s.i.=6894.76 Pa). Each residue was dissolved in 0.2 ml of ACN–MeOH (70:30) and transferred to 0.3-ml glass inserts with spring (Sunbroker, SC, USA), which were placed in HPLC vials (Sunbroker) for Waters autosampler Model 717, and 150-μl portions injected into the equilibrated HPLC system.

2.2.3. Chromatographic systems

The mobile phase was prepared by mixing 560 ml of ACN, 300 ml of MeOH, 140 ml of methylene chloride, 2.0 ml of glacial acetic acid and 2.0 ml of triethylamine. It was degassed before use by passing helium through it for a few minutes, followed sonication under vacuum. The HPLC system consisted of a Chirobiotic T, 250×4.6 mm, 5 μm column. An injection volume of 150 μl was delivered by a Waters Model 717 autosampler. The mobile phase was pumped at 2.5 ml/min by a Waters Model 510 solvent delivery system. The effluent was monitored by a Perkin-Elmer LC 240 fluorescence detector with excitation and emission wavelengths of 225 and 310 nm, respectively, (Response 5, Fix Factor 0.25 to 0.35). The output from the detector was collected and processed using the chromatographic software Waters Millennium system.

2.2.4. Assay validation

Standards of metoprolol and α-hydroxymetoprolol were prepared in human heparinized plasma (Biological Specialty) at concentrations of 100, 50, 25, 15, 7.5, 4, 2, 1 and 0.5 ng/ml for each of *R*-metoprolol and *S*-metoprolol. The internal standard was a 1000 ng/ml solution of atenolol in deionized water. Three 1-day runs and related experiments were performed to assess linearity, specificity, accuracy, stability, recovery, sensitivity and precision. Each daily run, designed to simulate an assay run, consisted of processing the set of standards containing both *R*- and *S*-metoprolol in the concentration range of 0.5 to 100 ng/ml each as described above, along with five sets of control samples at concentrations of 50, 15, 7.50, 2, 1 and 0.5 ng/ml. The concentrations of isomers of metabolite IIA and IIB in the all standards were the same as those of *R*- and *S*-metoprolol.

For all assays the peak height ratios of analyte to that of the internal standard were calculated. As would be done in an actual assay run, the slope and intercept of peak height ratio versus concentration for the first standard line were determined by linear least-squares regression with a weighting factor of $(1/\text{concentration}^2)$. The slope and intercept were then used to calculate the concentrations of the other standards and control samples in the run. The results were used to assess linearity, accuracy, precision and the lower limit of quantification (LLOQ).

2.2.5. Bioavailability study

The assay method was used to quantify the enantiomeric plasma concentrations of metoprolol and α-hydroxymetoprolol. This study was an open, randomized, fasting, single dose, four treatment, crossover study [24]. The plasma samples from human volunteers after administration of metoprolol were stored at −25°C until thawed for assay. Each typical analytical run consisted of a set of standards in the range 0.5/0.5 to 100/100 ng/ml of *R*-metoprolol/*S*-metoprolol combined with 1.0/1.0 to 100/100 ng/ml of IIA/IIB and samples from one subject

along with one set of control samples at concentrations of about 50/50/50/50, 15/15/15/15 and 7.5/7.5/7.5/7.5 ng/ml of *R*-metoprolol/*S*-metoprolol/IIA/IIB. The chromatographic data were collected and processed using Waters Millennium chromatography software. Least-squares linear regression of peak height ratio versus concentration (C) was performed for the standards using a weighting factor of 1/(concentration^2).

3. Results

3.1. Resolution

Representative chromatograms showing peaks for isomers of metoprolol, α-hydroxymetoprolol and the internal standard are shown in Fig. 2. *S*-Metoprolol, *R*-metoprolol, IIB and IIA eluted at about 5.9, 6.7, 7.3 and 8.2 min with chromatographic conditions of assay method A. The internal standard atenolol eluted as two isomers (*R* and *S*) at about 12.3 and 13.7 min. The metabolite *O*-desmethylmetoprolol (primary alcohol) coeluted with IIB and IIA under the chiral chromatographic conditions. However, no detectable levels of this primary alcohol were observed in the analysis of total α-hydroxymetoprolol. Also, it is an intermediate in the formation of ACMB in liver and no urinary concentrations have been observed in previous study [3]. Furthermore, the sum of IIB and IIA was equal to the total amount obtained during analysis of achiral α-hydroxymetoprolol. The acid metabolite (ACMB or H117/04) does not extract in the SPE method used for analysis of isomers of metoprolol and isomers of α-hydroxymetoprolol. For the achiral assay methods for α-hydroxymetoprolol the *O*-desmethylmetoprolol products are well resolved (primary alcohol and ACMB) which supports the above findings [23].

3.2. Linearity

Linearity was assessed by visual inspection of a plot of concentration versus the peak height ratio. The correlation coefficients, obtained from linear least-squares regression with 1/(concentration^2)

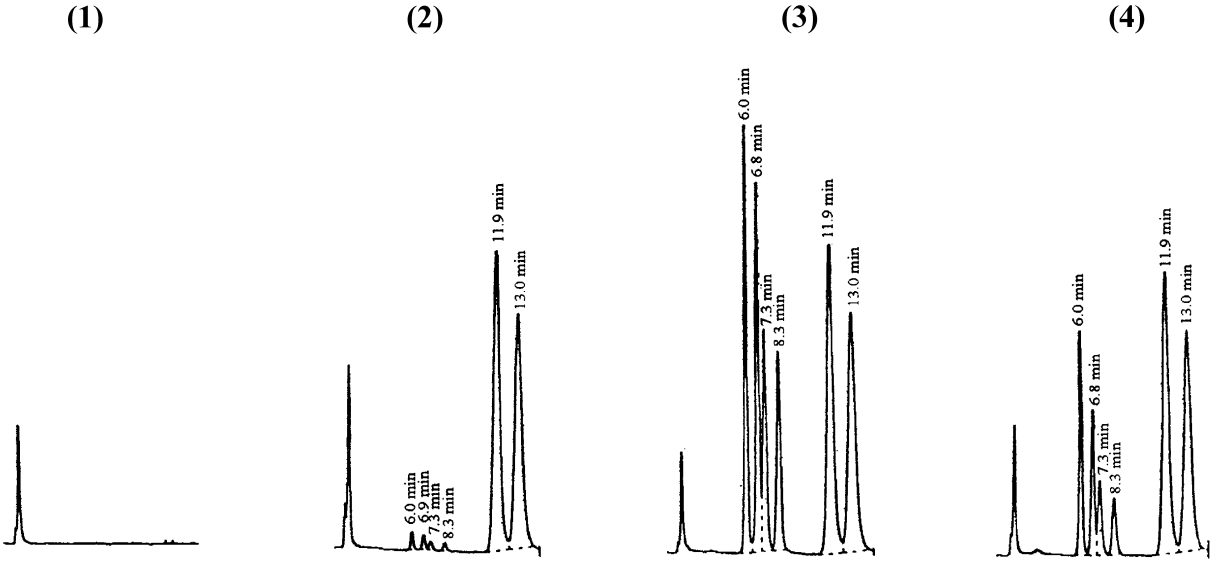

Fig. 2. Typical chromatograms of standards and unknown from a clinical chiral assay run for metoprolol and α-hydroxymetoprolol. Direct separation using Chirobiotic T bonded phase column (250×4.6 mm) and ACN–MeOH–$MeCl_2$–glacial acetic acid–triethylamine (56:30:14:2:2, v/v) mobile phase pumped at 2.2 ml/min. Retention times for *S*-metoprolol, *R*-metoprolol IIB and IIA were 6.0, 6.8, 7.3 and 8.3 min, respectively. Internal standard atenolol eluted with two isomeric peaks at 11.9 and 13.0 min. (1) Blank plasma extract, (2) standard containing 2 ng/ml each *R*-metoprolol and *S*-metoprolol and 2 ng/ml each IIA and IIB isomers, (3) standard containing 50 ng/ml each *R*-metoprolol and *S*-metoprolol and 50 ng/ml each IIA and IIB isomers, and (4) post dose sample from a typical patient.

weighting, were greater than 0.99. It is concluded, therefore, from these data and from the values obtained from back calculated concentrations of the standards that the calibration line conformed to a linear model over the range of 0.5 to 100 ng/ml for each isomer of metoprolol and 1 to 100 ng/ml for each isomer of α-hydroxymetoprolol (IIA & IIB).

3.3. Accuracy

Accuracy is defined as the ratio of the mean assayed concentration to that of the spiked concentration, expressed as a percentage. Table 1 summarizes the mean accuracy data for metoprolol and α-hydroxymetoprolol calculated for the standards in each daily validation run. All these mean intra-run accuracies were in the range of 96.2 to 114% for *R*-metoprolol, 94.0 to 111% for *S*-metoprolol, 90.2 to 110% for IIA, and 94.6 to 106% for IIB. When the accuracies from the individual daily validation runs were combined, the overall mean accuracies, measures of inter-run accuracy, were in the range of 97.2 to 106% for *R*-metoprolol, 97.9 to 106% for *S*-metoprolol, 90.4 to 104% for IIA and 97.7 to 104% for IIB.

3.4. Precision

Intra-run precision (Table 1) was assessed by calculating the relative standard deviation (RSD) of the control sample concentrations measured in each validation run. The mean intra-run precisions were all in the range of 2.2 to 12.0% for *R*-metoprolol, 2.1 to 11.1% for *S*-metoprolol, 1.9 to 14.5% for IIA, and 3.2 to 11.0% for IIB. The mean intra-run RSDs were all less than 14% except at low concentrations of analytes. When the values from the individual daily validation runs were combined the overall mean precisions, a measure of inter-run precision, were in the range of 3.2 to 10.7% for *R*-metoprolol, 3.8 to 9.8% for *S*-metoprolol, 6.6 to 10.2% for IIA, and 6.5 to 8.6% for IIB (Table 1).

3.5. Lower limit of quantification

The LLOQ was the lowest concentration that could be assayed with an accuracy of 85–115% and an RSD of no greater than 15%. The lowest standard processed in the 3-day validation meeting these requirements had a concentration of 0.5 ng/ml, accuracy 105% and an RSD of 7.6% for *R*-metoprolol, and accuracy of 97.9% and an RSD of 5.5%

Table 1
Precision and accuracy data for *R*-metoprolol, *S*-metoprolol, IIA [(1*R*2*S*, 1*R*2*R*)-α-hydroxymetoprolol] and IIB [(1*S*2*S*, 1*S*2*R*)-α-hydroxymetoprolol] assay validation

Concentration (ng/ml)	*R*-Metoprolol				*S*-Metoprolol			
	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*
50.0	48.6	3.16	97.1	15	49.7	4.26	99.5	15
15.0	14.9	3.94	99.2	15	14.9	3.81	99.3	15
7.5	7.69	4.93	103	14	7.72	5.48	103	14
2.00	2.10	7.70	105	14	2.03	7.12	101	14
1.00	1.06	10.7	106	15	1.06	9.83	106	14
0.50	0.525	7.63	105	10	0.490	5.49	97.9	10
Concentration (ng/ml)	IIA [(1*R*2*S*, 1*R*2*R*)-α-hydroxymetoprolol]				IIB [(1*S*2*S*, 1*S*2*R*)-α-hydroxymetoprolol]			
	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*
51	52.5	6.63	103	15	53.2	6.46	104	15
15.3	15.7	6.95	103	15	15.7	7.64	103	15
7.61	7.93	7.87	104	14	7.54	7.10	99.1	14
2.04	1.96	8.95	96.1	13	1.99	8.56	97.7	13
1.02	0.922	10.2	90.4	11	1.05	7.76	103	12

for *S*-metoprolol at a concentration of 0.5 ng/ml. In the same manner, the LLOQ for IIA and IIB was found to be 1.0 ng/ml each with RSDs of 10.2 and 7.8%, and accuracies of 90.4 and 103%, respectively.

3.6. Selectivity

Selectivity was assessed by processing four different blank plasma matrices using the same SPE method as for sample preparation. No endogenous peaks at the retention time of isomers of metoprolol, α-hydroxymetoprolol or internal standard (atenolol) were observed in any of the matrices using any of the assay method.

3.7. Ruggedness

Ruggedness was assessed by determining the retention times of isomers of metoprolol, α-hydroxymetoprolol, and internal standard using different analytical runs with different batches of mobile phases using the respective assay methods. No significant differences in retention times were observed. Previous experiments with SPE columns in the achiral assay method using C_4 analytical column showed that three different lots of SPE columns gave no significant difference in results when used to assay the metoprolol in a plasma sample containing 400 ng/ml metoprolol and 201 ng/ml α-hydroxymetoprolol. The mean assayed concentrations for the 400 ng/ml metoprolol and 201 ng/ml α-hydroxymetoprolol standard assayed in triplicate for the three batches of C_2 SPE column were 391 ng/ml with 1.4% RSD for metoprolol and 199 ng/ml with 2.3% RSD for α-hydroxymetoprolol.

3.8. Recovery

The absolute recovery of metoprolol, α-hydroxymetoprolol and internal standard were assessed by comparing the peak areas of extracted plasma standards to those of unextracted standards. The recoveries of metoprolol and α-hydroxymetoprolol were between 95 and 110% at all concentrations.

3.9. Stability

Plasma samples spiked with metoprolol tartrate and α-hydroxymetoprolol were prepared when the clinical phase of study was started. These stability samples were stored with subject samples at −25°C and served as a check of drug stability during storage over a 1-year period. The samples were thawed immediately before analysis and processed according to validated procedures previously described. The recovery of metoprolol (*R* and *S*) were between 99 and 102% (RSD<5.2%) and that of α-hydroxymetoprolol (IIA and IIB) were between 98 and 98.9% (RSD<7.0). The metoprolol and α-hydroxymetoprolol were found to be stable for at least 1 year at −25°C

3.10. Isomer peak identification

A high metabolizing subject was administered 11 mg of *R*-metoprolol HCl (99% pure) oral solution after overnight fasting. Urine samples were collected at 0 (pre-dose), 1, 2, 3, 6, 9, 14 and 18 h post-dosing and the collected urine volume was measured at each time. Samples were stored at −25°C until assayed. Urine samples were assayed using both the assay methods. *R*-metoprolol solution gave a single peak (6.8 min) when chromatographed. Analysis of sample resulted in peak single peak for IIA (8.3 min). There was no inversion of *R*-isomer to *S*-isomer since urine extracts showed only a single peak at the retention time of about 6.8 min for *R*-metoprolol.

3.11. Bioavailability study

Precision and accuracy data are presented in Table 2. A plasma concentration vs. time profile for each isomer following a single 100 mg dose of metoprolol is presented in Fig. 3. This profile highlights the sensitivity of the assay as well as its utility since it is possible to follow the time course of each enantiomer after a single dose. All of the results reported as analyte base concentration.

4. Discussion

Direct separation is preferred over indirect separation as it doses not require the additional step of chemical modification of analyte. It provided adequate resolving power as it separated metoprolol enantiomers with separation factor of >3. Direct separation using a Cyclobond III column resolved

Table 2
Precision and accuracy data for *R*-metoprolol, *S*-metoprolol, IIA [(1*R*2*S*, 1*R*2*R*)-α-hydroxymetoprolol] and IIB [(1*S*2*S*, 1*S*2*R*)-α-hydroxymetoprolol] assay in bioavailability study

Concentration (ng/ml)	*R*-Metoprolol				*S*-Metoprolol			
	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*
50.0	50.5	6.59	101	25	50.2	6.44	100	25
15.0	14.8	4.59	99.0	23	14.7	5.52	98.2	23
7.50	7.43	5.15	99.1	22	7.41	5.39	98.8	22
Concentration (ng/ml)	IIA [(1*R*2*S*, 1*R*2*R*)-α-hydroxymetoprolol]				IIB [(1*S*2*S*, 1*S*2*R*)-α-hydroxymetoprolol]			
	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*	Mean (ng/ml)	RSD (%)	Accuracy (%)	*n*
50.1	49.0	8.50	97.8	25	48.9	8.48	97.6	25
15.3	14.4	8.04	96.2	23	14.3	7.74	95.3	23
7.61	7.41	8.65	98.8	22	7.24	6.21	96.5	22

three enantiomers of α-hydroxymetoprolol. However, the fourth enantiomer remained unresolved even with temperature and/or mobile phase changes. A loss of resolution was observed between α-hydroxymetoprolol and metoprolol with the use of nonaqueous mobile phases due to possible cavity shrinkage. It was demonstrated that addition of small amounts of water to polar mobile phase restored baseline separation.

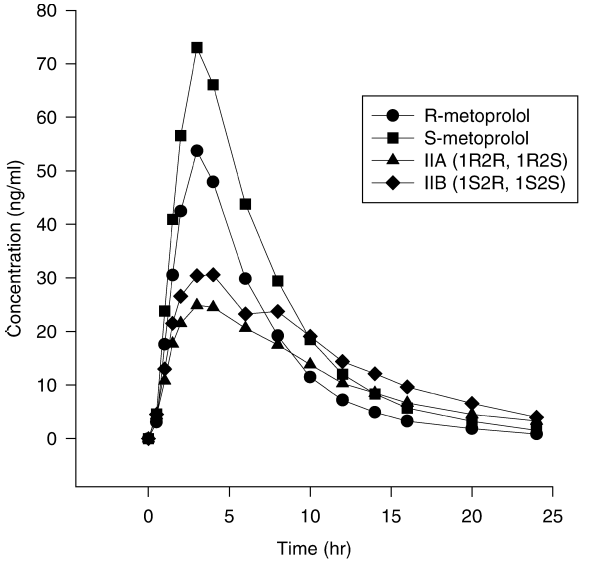

Fig. 3. *R*-Metoprolol, *S*-metoprolol, IIA (1*R*2*R*, 1*R*2*S*) and IIB (1*S*2*R*, 1*S*2*S*) plasma concentrations from a representative subject after the administration of an metoprolol extended release formulation.

Antibiotic bonded phases, a new generation of chiral columns, displayed remarkable resolution for β-blockers with a short analysis time. It exhibited specificity problems with compounds of similar structures (α amino alcohols) in the polar organic mode (MeOH, ACN, glacial acetic acid) since a number of different amino alcohols eluted very close to each other. This problem was easily solved by the addition of different relatively nonpolar organic mobile phase modifiers such as hexane and methylene chloride. Methylene chloride was used in the enantiomeric separation of metoprolol and its metabolites as a mobile phase modifier.

Recently enantiomer assays have been developed to quantify metoprolol in plasma or urine, however these methods do not measure the α-hydroxymetoprolol [20,21]. In addition, the sensitivity of the methods are 5 and 25 ng/ml, respectively [20,21]. Whereas the assay reported herein quantifies both the parent and α-hydroxymetoprolol metabolite and the level of sensitivity is 0.5 ng/ml. Another method has been developed to quantify the enantiomers of metoprolol, α-hydroxymetoprolol and its acid metabolite. However this method requires the use of four different stationary phases.

Stereospecific assays for the determination of enantiomers of metoprolol, and α-hydroxymetoprolol in human plasma have been developed, validated and

used successfully in bioavailability studies in which 100 mg doses of various formulations of metoprolol tartrate were administered to normal human volunteers [24]. The method presented here represents an improvement over several previously published methods to provide a rugged, sensitive and efficient method to analyze enantiomers of metoprolol and α-hydroxymetoprolol (IIA and IIB) in a large number of plasma samples with short sample analysis time. The SPE procedure used provides purer extracts, permitting greater sensitivity to be achieved. This greater sensitivity was needed to assay the low plasma concentrations expected after administration of extended release formulations. High reproducible recoveries were achieved in a single step and did not require the use of the large volumes of organic solvents used in liquid–liquid extractions. The natural fluorescence of metoprolol was used to attain the required sensitivity without derivatization and did not require a large volume of plasma sample for assay.

In the bioavailability studies, the assay performed to the specifications obtained during validation and no difficulties were encountered in the analysis of a large number of samples. The results were used to generate pharmacokinetic profiles and to calculate the pharmacokinetic parameters for metoprolol and metabolite stereoisomers.

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