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Solid-phase extraction and direct high-performance liquid chromatographic determination of metoprolol enantiomers in plasma

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ABSTRACT

A method is described by which underivatized metoprolol enantiomers in plasma can be quantitated by high-performance liquid chromatography with fluorescence detection. Samples are prepared for injection using a simple solid-phase extraction procedure which is essentially 100% efficient for all analytes. The metoprolol enantiomers are resolved using a cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phase and a hexane–ethanol–diethylamine mobile phase. Samples were tested for adaptability to autoinjection and found to be stable for at least 16 h after reconstitution in mobile phase. The automated method was determined to be precise and accurate for enantiomer concentrations as low as 10 ng base per ml and was successfully employed in a pharmacokinetic investigation.

INTRODUCTION

Metoprolol is a β-adrenoceptor antagonist widely used in the treatment of hypertension, angina pectoris and a number of other cardiovascular diseases [1]. Like other β-adrenoceptor blockers, metoprolol has an asymmetric center in its side-chain, and the commercially available product is actually a racemic mixture of two enantiomers. These enantiomers differ significantly in their pharmacodynamic and pharmacokinetic properties. *l*-Metoprolol is a much more potent β-adrenoceptor blocker (*i.e.* higher receptor affinity) than *d*-metoprolol [2]. Hepatic metabolism of metoprolol is largely dependent on a specific cytochrome P450 enzyme (debrisoquin hydroxylase or P450IID6) which is genetically regulated and polymorphically distributed in humans [3,4]. Stereoselective differences in metabolism occur in extensive metabolizers (approximately 90–95% of the Caucasian population), which lead to higher plasma concentrations of *l*-metoprolol than *d*-metoprolol [3,4]. As such, determination of metoprolol enantiomer plasma concentrations is essential to a proper understanding of the clinical pharmacokinetics and pharmacodynamics of metoprolol.

High-performance liquid chromatographic (HPLC) methods which involve

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derivatization of metoprolol enantiomers have been previously reported [5–7]. The diastereomers produced by these methods can be resolved by achiral HPLC, but sample preparation can be rather laborious. Development of chiral stationary phases (CSPs) has allowed the direct resolution of metoprolol enantiomers without the need for prior derivatization. Persson *et al.* [8] employed α_1-acid glycoprotein as the CSP to separate metoprolol enantiomers. Protein columns are usually less stable and suffer from relatively rapid loss of resolution when biologically based samples are injected. Although the more recently developed protein columns have been improved, Persson *et al.* [8] reported that their column required reactivation after only 150–200 injections.

Okamoto *et al.* [9] demonstrated that a CSP consisting of cellulose tris(3,5-dimethylphenylcarbamate) could resolve the enantiomers of β-adrenoceptor blockers. This same CSP, commercially available as the Chiralcel OD column (Daicel Chemical Industries, New York, NY, U.S.A.), was used in our laboratory to develop an assay for propranolol enantiomers in plasma [10]. Subsequently, other similar methods have been developed with the same column for the determination of metoprolol enantiomers [11–13]. A simple liquid–liquid extraction procedure was used by Rutledge and Garrick [11]. However, our experience and that of others [12,13] indicates that endogenous substances can interfere with the peaks of interest. In order to remove these interfering peaks, Straka *et al.* [13] added a second liquid–liquid extraction, whereas Ching *et al.* [12] added a solid-phase extraction.

In this report we describe an automated assay for underivatized metoprolol enantiomers using a simple solid-phase extraction. The assay is applied to the study of metoprolol pharmacokinetics.

EXPERIMENTAL

Materials

Racemic metoprolol tartrate, *d*-metoprolol · HCl, *l*-metoprolol · HCl, and metoprolol tartrate tablets (Lopressor) were provided by Ciba-Geigy (Summit, NJ, U.S.A. and Basel, Switzerland). The internal standard, *d*-propranolol · HCl, was provided by Wyeth-Ayerst (Princeton, NJ, U.S.A.). Methanol used in the extraction procedure (Burdick and Jackson, Muskegon, MI, U.S.A.) and hexane for the mobile phase (Mallinckrodt, St. Louis, MO, U.S.A.) were HPLC grade. Water was supplied by a Millipore Milli-Q water purification system (Bedford, MA, U.S.A.). Triethylamine (Sigma, St. Louis, MO, U.S.A.) and N,N-diethylamine (Fisher Scientific, Fairlawn, NJ, U.S.A.) were reagent grade. Ethanol was USP grade and supplied by Florida Distillers (Lake Alfred, FL, U.S.A.). The 3-ml solid-phase extraction columns which contain 200 mg of octadecylsilane packing were purchased from Analytichem International (Harbor City, CA, U.S.A.). Glass autosampler vial inserts were obtained from Sunbrokers (Wilmington, NC, U.S.A.).

Apparatus

The chromatography system consisted of a constant-flow reciprocating pump (Constametric III, LDC/Milton Roy, Riviera Beach, FL, U.S.A.) and fluorescence detector (Spectraflow 980, Kratos Analytical, Ramsey, NJ, U.S.A.). The guard and analytical (25 cm × 0.46 mm I.D.) columns were packed with cellulose tris(3,5-dimethylphenylcarbamate) polymer bonded to macroporous silica (Chiralcel OD, Daicel Chemical Industries). Samples were injected onto the column with the use of an automatic injector (SP8780 autosampler, Spectra Physics, San Jose, CA, U.S.A.) fitted with a 200-μl loop. The detector response was analyzed using a C-R5A Chromatopac integrator (Shimadzu, Columbia, MD, U.S.A.).

Chromatographic conditions

The mobile phase consisted of hexane–ethanol–N,N-diethylamine (95:5:0.1, v/v) pumped at a flow-rate of 0.5 ml/min. The guard and analytical columns were kept at room temperature. Excitation wavelength of the detector was set at 220 nm and a 320-nm high-pass filter was employed as an emission wavelength cut-off filter.

Extraction procedure

Plasma samples (1 ml) were placed in culture tubes and 15 ng of *d*-propranolol base (30 μl of a 0.5 ng base per μl methanol solution) were added. The samples were vortex-mixed for 15 s and applied to octadecylsilane solid-phase extraction columns which had been previously conditioned with 3 ml of methanol, followed by 3 ml of water. The columns were not allowed to dry before the addition of the plasma. After passing the plasma through the columns, the latter were rinsed with 3 ml of water, followed by 1 ml of 50:50 (v/v) methanol in water. Metoprolol and the internal standard were eluted into glass culture tubes with two aliquots of 500 μl of methanol containing 0.1% triethylamine (v/v). The eluted samples were then placed in a 30°C water bath and evaporated to dryness under nitrogen. Each sample was reconstituted with 150 μl of mobile phase, vortex-mixed for 30 s, and transferred to a 200-μl autosampler vial insert. Of each sample, 100 μl were automatically injected onto the column.

Extraction efficiency was determined by comparing the mean peak area obtained after six injections of 100 ng base of each metoprolol enantiomer in mobile phase with peak areas obtained after injection of five extracted plasma samples which had been spiked to a concentration of 150 ng base per ml of each enantiomer. Because only a portion of the available sample volume was injected, an adjustment was made to reflect the amount of drug present in the total volume of the extracted sample.

Calibration and reproducibility

Standard curves were constructed by injecting extracted plasma stocks spiked to concentrations of 400, 200, 100, 50, and 20 ng base per ml of each metoprolol

enantiomer (1000 nmol/l is equivalent to 267.38 ng base per ml). The standard curves were evaluated using weighted linear regression analysis, with each standard peak-area ratio weighted to the inverse of its variance. The variances were estimated from ten replicate determinations for each standard. The coefficient of determination (r^2), calculated as the regression sum of squares/total sum of squares, was used as a measure of goodness of fit.

Between-day variability was determined with control samples (between 300 and 10 ng base per ml of each enantiomer) which were extracted and injected daily for five days. Similarly, within-day variability was assessed with five repeated injections, on the same day, for each of the six control samples. The metoprolol enantiomer concentrations were calculated from the single standard curve run the same week. Calibration curves were also run each day for ten days to determine the reproducibility of the peak-area ratio for each standard. The stability of reconstituted samples awaiting injection by the autosampler was evaluated with nine extracted control samples injected over 16 h.

Pharmacokinetic evaluation

One healthy male subject received a single 200-mg oral dose of racemic metoprolol tartrate after an overnight fast. The subject was previously phenotyped and was determined to be an extensive metabolizer of dextromethorphan (marker for the debrisoquin hydroxylase or P450IID6 enzyme). Blood samples were collected by separate venipunctures at 0, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after the dose. After centrifugation, plasma was collected and stored in polypropylene tubes at −20°C until analysis.

RESULTS AND DISCUSSION

The hexane–ethanol–diethylamine mobile phase separated *d*-metoprolol from *l*-metoprolol with a mean stereochemical resolution (R_s) of 1.99 and mean selectivity (α) of 1.19. Average capacity factors (k') for *d*-metoprolol, *l*-metoprolol, and *d*-propranolol were 2.06, 2.46, and 4.34, respectively. The above parameters (R_s, α and k') were determined by standard methods [14]. Elution order was confirmed with injection of the individual metoprolol enantiomers. Representative chromatograms are shown in Fig. 1 and demonstrate that endogenous compounds did not interfere with any of the peaks of interest.

The standard curves ($n = 10$) were linear over the concentration range used, with r^2 values always exceeding 0.988 for *d*-metoprolol and 0.990 for *l*-metoprolol. The coefficients of variation (C.V.) for the peak-area ratios of the different standards ranged from 3.46 to 5.76% for *d*-metoprolol and 3.80 to 6.81% for *l*-metoprolol.

Table I summarizes the results of the within-day and between-day measurements of accuracy and precision. The efficiency of the solid-phase extraction was 99.6% for *d*-metoprolol (C.V. − 4.78%) and 99.2% for *l*-metoprolol (C.V. =

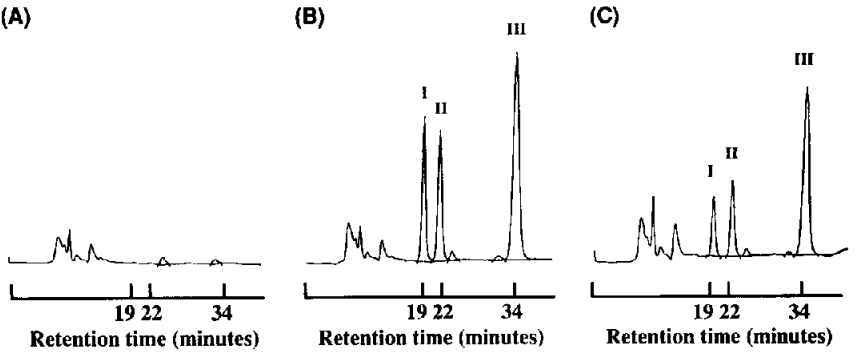

Fig. 1. Representative chromatograms for (A) an extracted blank plasma sample, (B) a plasma sample spiked with 150 ng base per ml of each metoprolol enantiomer, and (C) a subject's plasma sample 1.5 after oral administration of a 200-mg dose of racemic metoprolol tartrate (measured concentrations are 73 ng base per ml for *d*-metoprolol and 104 ng base per ml for *l*-metoprolol). Peaks: I = *d*-metoprolol; II = *l*-metoprolol; III = *d*-propranolol.

4.77%) at 150 ng base per ml of each enantiomer. The limit of detection, defined as a signal-to-noise ratio of 4:1, was 4 ng base per ml for each enantiomer. Reconstituted control samples which were injected over a period of 16 h showed no systematic evidence of degradation and a C.V. of 3.26%.

TABLE I

ANALYTICAL PRECISION AND ACCURACY IN THE DETERMINATION OF *d*- AND *l*-METOPROLOL FROM SPIKED PLASMA SAMPLES

See text for experimental conditions.

Concentration added (ng base/ml)	*d*-Metoprolol		*l*-Metoprolol	
	Concentration observed (mean) (ng base/ml)	C.V. (%)	Concentration observed (mean) (ng base/ml)	C.V. (%)
Within-day variation (n = 5)				
300	298.55	0.98	301.48	1.06
150	142.81	4.34	142.12	2.76
75	76.34	1.17	74.23	0.84
30	29.69	2.02	29.57	2.69
14	14.66	5.49	14.40	4.51
10	10.88	4.07	10.46	3.45
Between-day variation (n = 5)				
300	281.39	3.62	284.15	4.22
150	141.25	1.78	141.06	1.78
75	75.91	3.15	74.68	2.48
30	29.61	4.71	28.66	1.33
14	14.86	8.49	14.40	4.07
10	10.27	7.72	10.46	6.56

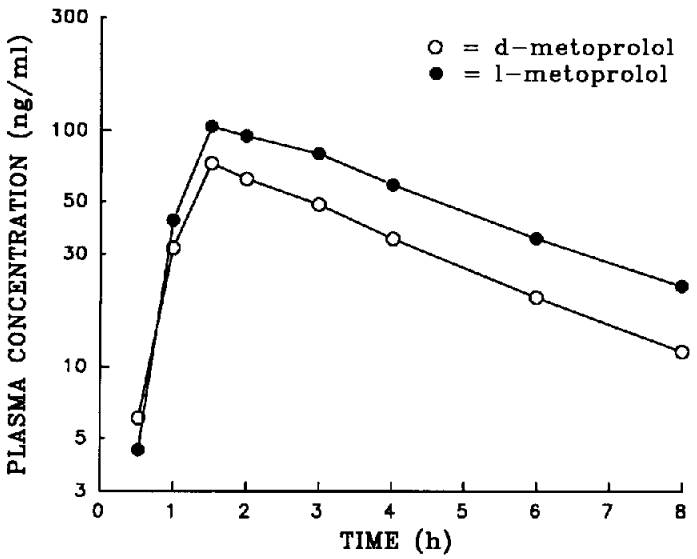

Fig. 2. Plasma enantiomer concentration-time curves after oral administration of a 200-mg dose of racemic metoprolol tartrate to a healthy male subject with the extensive metabolizer phenotype.

Plasma concentration–time curves for metoprolol enantiomers are shown in Fig. 2. The apparent oral clearances, calculated as dose divided by area under the curve from time 0 to infinity, were 62.6 and 37.9 ml/min/kg for *d*- and *l*-metoprolol, respectively. The terminal half-lives were 2.5 h for *d*-metoprolol and 2.8 h for *l*-metoprolol. These results are consistent with previously reported values for subjects with the extensive metabolizer phenotype [3].

The method we describe to quantitate metoprolol enantiomers in plasma has significant advantages over other published methods. Our extraction method is fast, relatively inexpensive, and avoids exposure to hazardous organic solvents. The extracted samples seem to be relatively free of particulate matter and other materials which are trapped on the chromatographic system by the guard column. After >750 injections, the majority of which were extracted plasma samples, our system maintains a stable pressure of <34.5 bar (<500 p.s.i.).

We determined that the peaks of interest are more narrow with ethanol compared to 1-propanol or isopropanol as the mobile phase modifier. This allows adequate resolution of metoprolol enantiomers, despite the modest selectivity, and produces peaks that are symmetrical. Our mobile phase may avoid the problems associated with the broad, tailing peak shape of *l*-metoprolol reported by other investigators who used the same CSP and a hexane–isopropanol mobile phase [13]. Although other investigators [11,13] have used an excitation wavelength around 275 nm, we observed that, under our assay conditions, peak heights were increased four-fold at a wavelength of 220 nm with a deuterium

lamp source. Fluorescent yield was not affected by the mobile phase modifier (*i.e.* ethanol, 1-propranol, or isopropanol) but it was decreased when the diethylamine concentration approached 1%. Consequently, we elected to use an excitation wavelength of 220 nm and 0.1% diethylamine in the mobile phase, similar to the conditions employed by Ching *et al.* [12]

The standard curve concentrations were chosen to encompass the full range of metoprolol concentrations that are expected in a clinical pharmacokinetics study. However, the highest concentration point on the curve strongly affected the results of the unweighted linear least-squares regression used to evaluate the standard curve. This resulted in larger relative errors at low concentrations consistent with statistical theory and the experience of other investigators [13]. We found that weighted linear least-squares regression reduced this error. In order to increase the throughput of samples, we determined whether a single weekly set of standards could be used for the evaluation of controls and subjects' samples run during that same week. The accuracy and precision results from Table I demonstrate that weekly standard curves provide satisfactory results. Samples with enantiomer concentrations as low as 10 ng base per ml can be quantitated with C.V.s less than 8.5%.

In conclusion, the results described in this paper and subsequent investigations in our laboratory [15] indicate that this assay is suitable for studies of metoprolol enantiomer pharmacokinetics. The method is very simple because it involves direct resolution of metoprolol enantiomers, solid-phase extraction from plasma, and allows for automatic injections to maximize efficiency.

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