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Note

Reversed-phase high-performance liquid chromatographic assay to quantitate diastereomeric derivatives of metoprolol enantiomers in plasma

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It has been demonstrated that because of differences in receptor affinity and enzyme-mediated clearance, enantiomeric forms of a drug may have different or even opposing activities [1]. Since many drug products are racemic mixtures, difficulties may arise in studies in which drug concentration is to be correlated with pharmacologic effect unless concentrations of individual isomers are measured. This is particularly relevant for β-adrenergic receptor antagonists, since data from clinical [2–4] studies have demonstrated markedly different pharmacologic activity for the (+)- and (−)-isomers of a diverse array of β-blocker effects. Thus it is important to establish and simplify methods of analysis which are enantiospecific at clinically relevant concentrations in biological fluids.

There have been several publications reporting procedures for the determination of enantiomer concentrations of various β-adrenergic antagonists by direct [5,6] and indirect [7–9] methods. Currently available procedures for the quantitation of individual metoprolol isomers either involve tedious derivatizations

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[8,9] or have not been applied to drug concentration determination in plasma [5]. A reversed-phase high-performance liquid chromatographic (HPLC) assay is presented here which extends the sensitivity and simplifies the determination of the concentration of the individual metoprolol enantiomers in plasma from humans treated with the racemic mixture.

EXPERIMENTAL

Reagents

HPLC-grade methylene chloride, acetonitrile and tetrahydrofuran were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Sodium hydroxide (2 *M*) and glacial acetic acid were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). (±)-Metoprolol (+)-tartrate, triethylamine (TEA), phosphoric acid and heptanesulfonic acid sodium salt (HSA) were obtained from Sigma (St. Louis, MO, U.S.A.). The individual metoprolol enantiomers were a gift from Ciba-Geigy (Summit, NJ, U.S.A.). The derivatization reagent, 2,3,4,5-tetra-O-acetyl-β-*d*-glucopyranosyl isothiocyanate (GITC), was purchased from Polysciences (Warren, PA, U.S.A.). The optical purity of GITC was determined indirectly by chromatographically analyzing the reaction products of the reagent with the individual isomers of metoprolol.

Solutions of the individual isomers as well as of racemic metoprolol were prepared to a concentration of 100 μg/ml free base in distilled, deionized water. These bulk metoprolol solutions were frozen and aliquots were thawed as needed, then stored at 4°C. GITC was dissolved in acetonitrile to a concentration of 100 μg/ml and stored at 4°C when not in use.

Instrumentation

All chromatography was performed on a 10-μm Waters μBondapak C_{18} column, 300 mm × 4.6 mm I.D. The enantiomers were eluted with acetonitrile–water (48:52) which contained 0.1% TEA. The pH of the mobile phase was adjusted to 3.5 with phosphoric acid. The flow-rate was 2 ml/min. Detection of the individual diastereomeric derivatives was achieved at 222 nm with a Waters Model 450 UV detector. Detector sensitivity was set to 0.01 a.u.f.s. and a time constant of 1 s was employed.

Preparation of plasma standards

To a known volume of drug-free plasma, an aliquot of racemic metoprolol solution was added to prepare plasma standards containing 10–1000 ng/ml of each enantiomer (i.e. 20–2000 ng/ml total metoprolol).

Extraction and derivatization procedure

A 1.0-ml aliquot of plasma standard or sample was added to a borosilicate glass tube and 50 μl of 2 *M* sodium hydroxide and 4 ml of methylene chloride were added to each tube. The tubes were then vortexed vigorously for 15 s and centrifuged for 5 min at approximately 2000 *g* to achieve phase separation. The upper, aqueous phase was discarded and 3.6 ml of the organic layer were transferred to

a clean borosilicate tube. The methylene chloride was evaporated under a stream of nitrogen at room temperature. The residue was then reacted with GITC by adding 200 μl of the GITC solution. The reaction was allowed to proceed for 30 min at room temperature. After the reaction was complete, the acetonitrile was evaporated under a stream of nitrogen at room temperature and the sample was reconstituted in 100 μl of mobile phase. An 85-μl aliquot of this final solution was injected.

Percentage recovery

In order to estimate the fraction of metoprolol that undergoes derivatization, samples were prepared from plasma using the above procedure. The starting concentrations of drug were made much higher than those found in most clinical samples since higher drug concentrations would be expected to provide a more difficult test for the completeness of the derivatization reaction. These samples were assayed for unreacted metoprolol by ion-pair chromatography [10]. Peak heights were compared to peak heights of samples prepared identically, except that acetonitrile is substituted for GITC solution in the derivatization step.

Hepatic microsomal metabolism

Microsomes were obtained from male Sprague–Dawley rats pretreated with 100 mg/kg phenobarbital daily for five days. This preparation was then diluted to a total protein concentration of 19 mg/ml. The diluted preparation was fortified by adding NADP (3.3 mg/ml), glucose-6-phosphate (4.8 mg/ml) and glucose-6-phosphate dehydrogenase (4.3 U/ml). Tris–HCl buffer was prepared to 100 m*M*. The Tris buffer also contained 300 m*M* potassium chloride and 10 m*M* magnesium chloride hexahydrate. The pH of the buffer was adjusted with hydrochloric acid to 7.5.

A preincubated suspension was prepared from 200 μl of the microsome suspension, 200 μl of cofactor solution, 750 μl of Tris buffer and 750 μl of water. After incubating this solution for 2 min at 37 °C, an aliquot of drug solution of concentration 1 m*M* was added and the samples were incubated for 30 min at 37 °C with constant shaking (aliquots of 100 μl of drug solution were used). The solutions contained either (+)-metoprolol, (−)-metoprolol or racemic metoprolol. The reaction was terminated by the addition of 500 μl of 2.5 *M* sodium hydroxide. Aliquots of 1 ml of the reaction mixture were extracted into 4 ml of methylene chloride and processed as per the above procedure. The dried samples were redissolved in acetonitrile containing 1 mg/ml GITC.

RESULTS

The separation of diastereomeric derivatives of (+)-metoprolol from (−)-metoprolol enabled quantification of individual isomer concentrations. Fig. 1 shows the chromatograms obtained from blank serum (A), the individual isomers (C and D) and separation of isomers from serum sampling containing racemic metoprolol (B). The separation of the individual isomers, extracted from the plasma containing racemic metoprolol, was complete, with the (+)-isomer peak

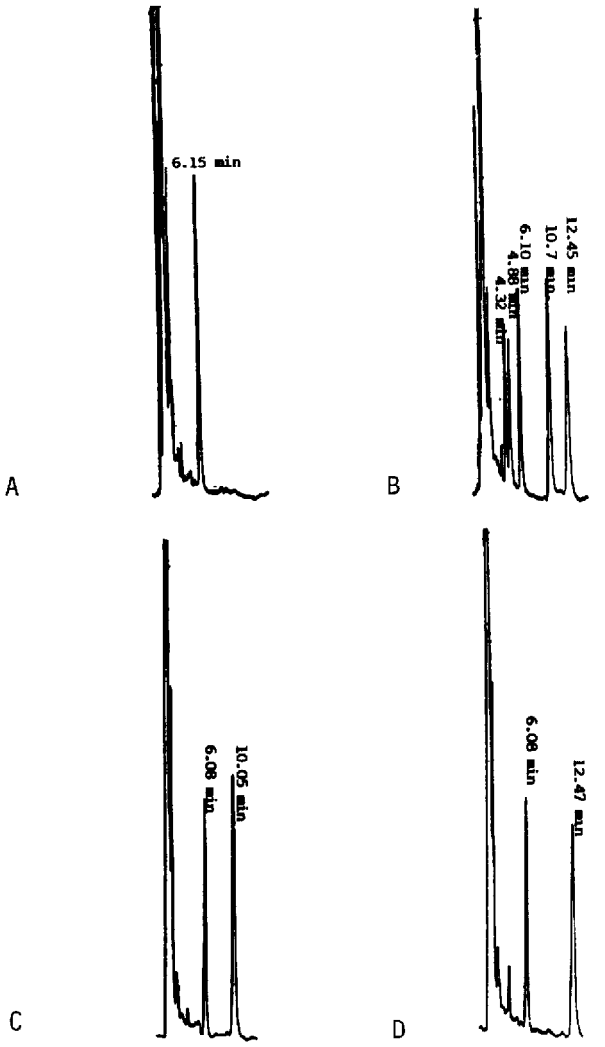


Fig. 1. Resolution of the isomer of metoprolol and α-hydroxymetoprolol as their GITC derivatives. Column, ODS-10, 250 mm × 4.6 mm I.D. Mobile phase, acetonitrile–water (48·52) with 0.1% TEA. The pH is adjusted to 3.5 with phosphoric acid. Flow-rate is 2 ml/min and UV detection is carried out at 222 nm. (A) Blank plasma with GITC; the peak at 6.15 min results from GITC. (B) Plasma spiked with 1000 ng/ml each of α-hydroxymetoprolol (retention times 4.32 and 4.88 min) and racemic metoprolol (retention times 10.7 and 12.45 min). (C) Plasma spiked with 500 ng/ml (−)-metoprolol (retention time 10.05 mın). (D) Plasma spiked with 500 ng/ml (+)-metoprolol (retention time 12.47 min).

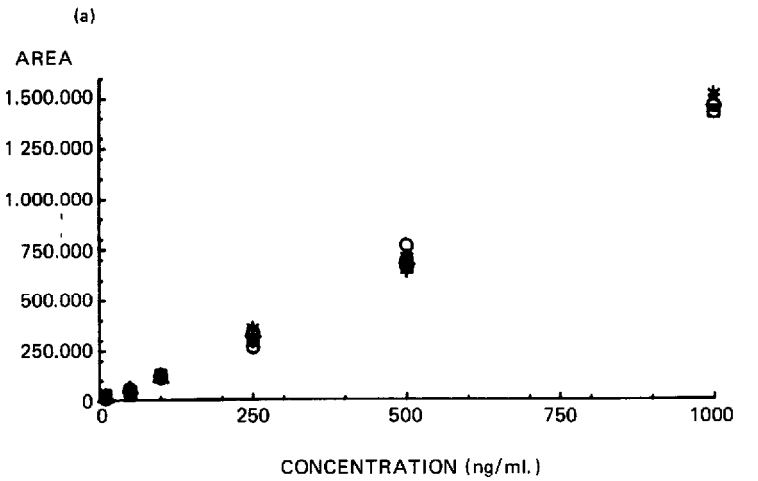
occurring at 12.45 min and the (−)-isomer occurring at 10.07 min. The enantiomer concentration that results in a signal-to-noise ratio of 3:1 is approximately 10 ng/ml for the (−)-isomer and 10 ng/ml for the (+)-isomer.

To assess the reproducibility of this method, six standard curves were prepared, each curve containing calibration samples and quality control samples. Table I contains the mean (±S.D.) slope and intercept data of these studies and Fig. 2a

TABLE I

SUMMARY OF REGRESSION ANALYSIS

Compound	Slope (mean ± S.D.) (area ml/ng)	Intercept (mean ± S.D.) (area/10^{-3})
(−)-Metoprolol	1477 ± 44	−26.810 ± −9.664
(+)-Metoprolol	1395 ± 38	−28.708 ± −7.383



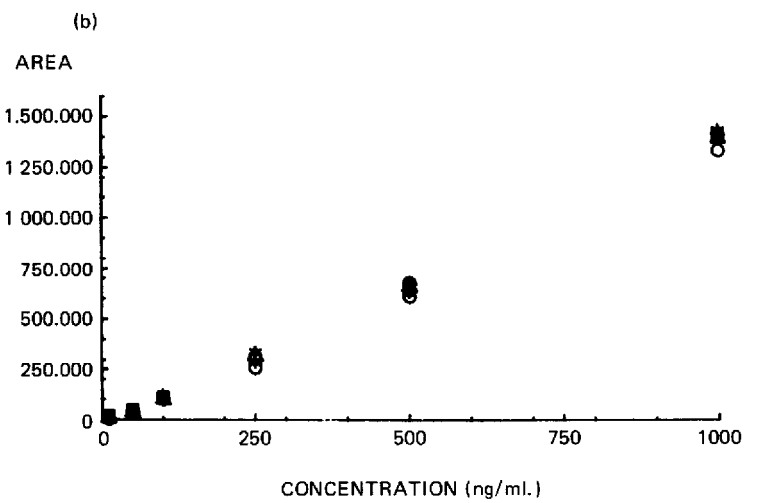


Fig. 2. (a) Six superimposed standard curves (−)-metoprolol; (b) six superimposed standard curves (+)-metoprolol.

and b provide a summary of the cumulative results of these six curves for each individual enantiomer.

As seen in Table II, at all concentrations tested, less than 2% of the metoprolol in the samples is recovered as unreacted metoprolol. Our extraction procedure for metoprolol from plasma is similar to the one found in a published metoprolol assay [10] in which the extraction efficiency is given as 89%.

Clinical application

Serum samples from two patients taking racemic metoprolol were analyzed using the stereoselective procedure. The results were compared to an analysis of these samples for metoprolol by a non-stereoselective assay [10]. As seen in Fig. 3, there is good agreement between racemic metoprolol concentrations and the sum of the individual isomer concentrations.

TABLE II

RESULTS OF THE RECOVERY EXPERIMENT

Concentrations represent racemic metoprolol as free base.

Initial concentration (ng/ml)	Concentration unreacted (ng/ml)	Percentage unreacted
500	8	1.6
2000	15	0.75
3000	23	0.7
4000	29	0.73

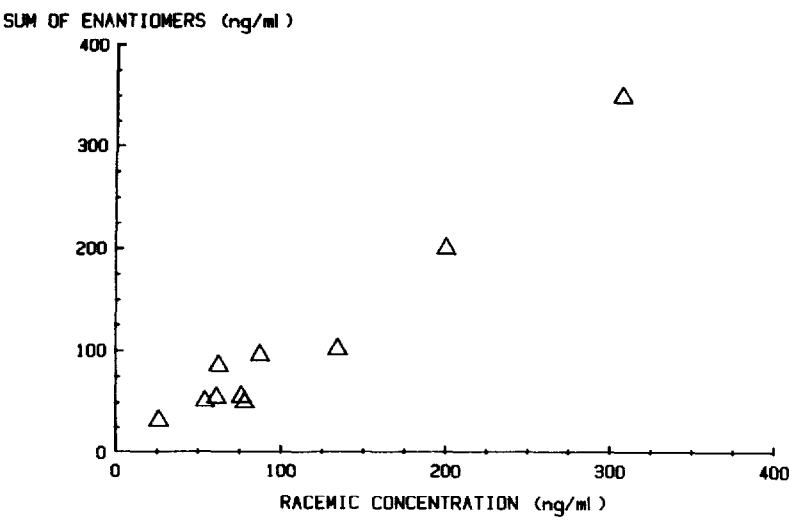


Fig. 3. Correlation of total concentrations from racemic metoprolol assay and enantiomer assay. The Pearson correlation coefficient is 0.977 and the slope of the line is 0.82.

DISCUSSION

The assay described provides a method to determine the concentration of individual metoprolol enantiomers from human serum. The separation between the diastereomeric derivatives is good ($R=1.72$, $\alpha=1.34$) and the data show that the assay is a reliable and reproducible predictor of enantiomer and total drug concentrations.

In order to establish that this procedure is selective for metoprolol, several other β-blockers were assayed. Atenolol, pindolol and propranolol all react when incubated with GITC under the conditions presented here. None of the resulting diastereomers could be mistaken for metoprolol. Atenolol eluted at 3.3 and 3.6 min, pindolol at 7.2 and 8.4 min and propranolol at 19.0 and 23.4 min.

This procedure is a modification of previously reported methods which are used either for non-biological samples [5] or urine samples at higher concentrations. An increase in the water fraction of the mobile phase allow data on metoprolol and α-hydroxymetoprolol to be collected simultaneously. The α-hydroxymetoprolol peaks can be seen in Fig. 1D at 4.32 and 4.88 min ($R=0.95$, $\alpha=1.28$). In order to establish the origin of each metabolite peak, microsomal metabolism was performed on each individual enantiomer of metoprolol and on racemic metoprolol. The resulting samples were assayed for diastereomeric derivatives of α-hydroxymetoprolol. It was determined that the first α-hydroxymetoprolol peak resulted from the metabolism of (−)-metoprolol and the second α-hydroxymetoprolol peak from the metabolism of (+)-metoprolol. Baseline resolution of the metabolite peaks can be achieved by increasing the aqueous portion of the mobile phase to 60%. This mobile phase would be impractical for clinical samples, however, since the increase in water necessitates an assay time of 30 min to resolve metoprolol.

Modification in the GITC concentration allows a lower limit of detection to be achieved because apparent impurities in this reagent yield extraneous peaks. A ten-fold dilution of GITC reduces interference from impurities and still results in almost complete reaction with metoprolol, thus allowing the assay to be utilized for therapeutic monitoring of clinically relevant plasma samples (Table II).

In summary, the procedure described offers a simple and reliable method for determining the concentrations of individual metoprolol isomers from plasma. The signal-to-noise ratio of 3:1 at 10 ng/ml indicates that the sensitivity of this assay is adequate for use in clinical studies designed to explore the relationship between drug plasma concentration and pharmacologic effect.

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