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STEREOSELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PROPRANOLOL ENANTIOMERS IN SERUM

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SUMMARY

A simple and sensitive stereoselective high-performance liquid chromatographic assay for the quantitation of propranolol enantiomers in serum is described. The method involves conversion of the propranolol enantiomers to diastereomeric urea derivatives by reaction with the chiral reagent (+)-phenylethylisocyanate, followed by chromatographic separation of the diastereomeric products. Conditions of the derivatization reaction were optimized to achieve rapid and quantitative yield with either of the enantiomers. Baseline resolution of the diastereomers was achieved on a reversed phase C_8 column with an isocratic mobile phase. Fluorescence detection afforded an absolute on-column detection limit of 100 pg. The assay has been applied to pharmacokinetic studies in humans and small laboratory animals.

INTRODUCTION

The non-selective β-adrenoreceptor antagonist, propranolol, has been used in the treatment of a variety of cardiovascular disorders. The available commercial dosage forms of propranolol contain a racemic mixture of the compound. Like many other β-adrenergic agents, the pharmacologic activity of propranolol is enantioselective. $S(-)$-Propranolol is a 100-fold more potent in β-adrenergic blocking activity than its optical antipode [1]. Recent studies have shown that the metabolism and disposition kinetics of propranolol are also highly stereoselective (see review by Walle [2]). Therefore, analytical procedures capable of resolving

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and quantitating the stereoisomers of propranolol enantiomers in biological fluids are needed for pharmacokinetic studies following administration of the racemic compound.

During the last decade, a variety of analytical methodologies for the resolution and quantitation of propranolol enantiomers have been developed. Earlier on, several radioimmunassays and radioreceptor assays were developed [3–6]. While these procedures are quite sensitive, they can measure only one enantiomer at a time. Also, cross-reactivity of propranolol metabolites was an ever present problem with protein binding assays.

Much of our knowledge concerning the stereoselectivity of propranolol metabolism was obtained through the use of stable isotope-labeled pseudoracemate coupled with gas chromatography–mass spectrometry (GC–MS) [2]. Even with the recent increase in availability of mass-selective detectors, the cost of pseudoracemate synthesis and GC–MS instrumentation is prohibitive for most laboratories. Therefore, a great deal of effort has been devoted to chromatographic means of resolution.

Resolution of propranolol enantiomers with chiral stationary phases has attracted considerable attention. There are two different approaches to the use of high-performance liquid chromatography (HPLC) chiral columns. Most of the earlier methods, especially those developed for the (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine phase (i.e., the Pirkle CSP 1), require conversion of the analytes to enantiomeric derivatives to achieve adequate resolution [7–9]. Direct chromatographic resolution of propranolol enantiomers can now be achieved with several of the recently developed chiral HPLC columns, such as the α_1-acid glycoprotein, cyclodextrins and the cellulose–tris(3,5-dimethylphenyl carbamate) phases [10–12]. A major drawback of the use of chiral stationary phase is the high cost of the commercial columns. Also, in most cases baseline resolution of propranolol enantiomers is only achieved with long elution times (>20 min), which renders the assays impractical for routine analysis requiring high throughput.

An alternative approach to chromatographic resolution of ionizable stereoisomers involves addition of optically active ion-pairing agents to the mobile phase. For example, (+)-10-camphorsulphonic acid was used in the separation of propranolol enantiomers [13]. Unfortunately, poor resolution has been a problem.

A long favored approach involves reacting propranolol enantiomers with a chiral derivatizing reagent, followed by chromatographic resolution of the diastereomeric products [14–16]. The derivatizing agent must meet stringent requirements as to chemical and optical purity, and should react with the stereoisomers in a rapid and quantitative fashion. Until recently, the major problem with the diastereomeric derivatizing approach had been the availability of optically pure chiral reagent. For example, one commercially available chiral reagent that has been proposed for propranolol, N-trifluoroacetyl-*S*(−)-prolyl chloride, was found to be contaminated with 4–15% of the *R*(+)-enantiomer [14].

In recent years a number of chiral isocyanate reagents have been developed for use with β-blockers [17–19]. These reagents have the advantage of being stereochemically stable. Many of these compounds are now available in high purity

form from commercial sources, e.g., 2,3,4,6-tetra-O-acetyl-β-*d*-glucopyranosyl-isothiocyanate, phenylethylisocyanate (PEIC), tri-O-acetyl-α-*d*-arabinopyranosylisothiocyanate, and $R(-)$-1-naphthylethylisocyanate. The most experience has been acquired with PEIC. The use of either $(+)$ or $(-)$-PEIC was first proposed by Thompson et al. [17]. These investigators showed that reaction with racemic propranolol resulted in the formation of diastereomeric urea derivatives which can be resolved by HPLC and GC. While the derivatization reaction was shown to be reproducible and quantitative, there is only limited experience in the application of this procedure to the assay of biological specimens. Wilson and Walle [20] reported the simultaneous determination of propranolol and 4′-hydroxypropranolol enantiomers in urine by silica gel HPLC after derivatization with $R(+)$-PEIC. This article reports the development of a reversed-phase HPLC assay capable of quantitating ng/ml levels of propranolol enantiomers in serum based on chiral derivatization with $R(+)$-PEIC.

EXPERIMENTAL

Chemicals

Racemic propranolol hydrochloride, $S(-)$-propranolol hydrochloride ($[\alpha]_D^{20}$: observed $-21.40°$ versus reported $-23.86°$, $C=1.0$ in ethanol), and $R(+)$-propranolol hydrochloride ($[\alpha]_D^{20}$: observed $+24.40°$ versus reported $+24.52°$) were obtained from Ayerst Labs. (New York, NY, U.S.A.). The internal standard, $(\pm)$-N-cyclopentyldesisopropylpropranolol (CDP), was provided by Pierce (Rockford, IL, U.S.A.). All organic solvents were of high-purity HPLC grade (American Burdick & Jackson Labs., Muskegon, MI, U.S.A.). $R(+)$-PEIC ($[\alpha]_D^{20}=+0.61°$, $C=1$ in benzene) was purchased from Fluka (Ronkonkoma, NY, U.S.A.).

Preparation of standards

A concentrated stock solution of CDP (60.9 μg/ml) was prepared in methanol. The methanolic solution was stable for about one month at $-4°$C. A working solution was prepared by diluting the CDP stock solution to a concentration of 600 ng/ml in water.

Standard solutions at various concentrations of racemic drug were prepared by diluting a stock solution of $(\pm)$-propranolol in dilute phosphoric acid (0.0122 *M*, pH 3.3). Calibrators were prepared by spiking blank human or rat serum with 10 μl of each standard solution to achieve final concentrations ranging from 6 to 96 ng/ml racemic propranolol (i.e., 3 to 48 ng/ml of each enantiomer).

Derivatization and extraction procedure

A scheme outlining the steps involved in sample preparation is presented in Fig. 1. Specimens or standards in 100-μl aliquots were placed in 13×100 mm Teflon-lined screw-capped and tapered glass centrifuge tubes (Kimble, IL, U.S.A.). A 10-μl volume of the working solution of CDP is added. Serum was alkalinized by the addition of 200 μl of 10% sodium bicarbonate, followed by the addition of 5 ml of diethyl ether. After shaking for 15 min with a blood mixer (20

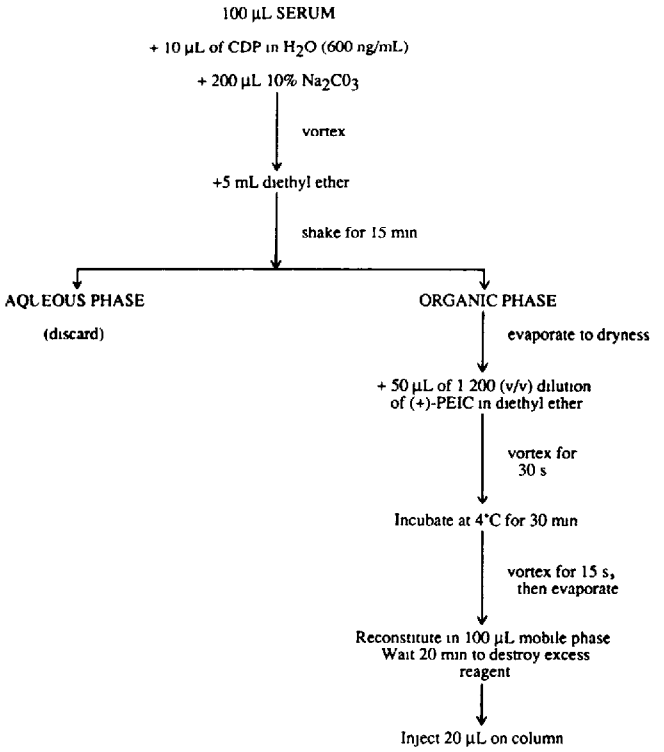

Fig. 1. Flow chart for the extraction and derivatization of propranolol enantiomers.

rpm) and centrifugation at 400 *g* for 5 min, the upper organic phase was transferred to a separate glass centrifuge tube. The organic phase was then evaporated to dryness under purified nitrogen at room temperature. *R*(+)-PEIC was diluted in diethyl ether (5 µl per ml). A 5-µl volume of this ethereal solution was added to each tube of serum extract. The tubes were vortexed vigorously for 30 s and then kept at 4°C for 30 min. Afterwards, the tubes were allowed to warm up to room temperature, and the reaction mixture was evaporated under nitrogen. The residue was reconstituted in 100 µl of mobile phase and left standing at room temperature for 20 min to destroy any unreacted *R*(+)-PEIC. A 20µl aliquot of the derivatized extract was then injected onto the column.

Instrumentation and chromatographic conditions

The chromatographic system consisted of a Waters Model 6000A high-pressure pump, a Rheodyne Model 7120 injector fitted with a 20-µl loop, and a Kratos FS-970 fluorescence detector (10-µl flow cell). The excitation wavelength was set at 220 nm. A 300-nm cut-off filter was placed in the emission beam. The time constant was set at 4 s and the output signal ranged between 0.1 and 0.2 µA.

Chromatographic separation was achieved with a 5-µm (25 cm×4.6 mm I.D.) Altex Ultrasphere C_8 reversed-phase column (Rainin Instruments, Westwood, NJ, U.S.A.). A guard column with 25–37 µm pellicular C_{18} packing (Co:Pell ODS) from Whatman Chemical Separations (Clifton, NJ, U.S.A.) was used. The mo-

bile phase consisted of methanl–isopropanol–methylene chloride–water (67:7.5:1:25.5). The mobile phase flow-rate was set at 0.7 ml/min.

Calibration

A set of serum standards was assayed along with each batch of samples. Calibration plots were constructed by linear least-squares regression of the peak-height ratio of the diastereomeric derivative of each propranolol enantiomer to one of the diastereomeric peaks of the racemic internal standard (the first-eluting peak) against the standard enantiomer concentration. The within-batch precision of the assay was checked at two different serum concentrations of propranolol enantiomers (6.3 and 52.5 ng/ml). Inter-day assay variation was assessed by replicate analysis of a control serum sample containing 25.4 and 50.7 ng/ml of each enantiomer in a racemic mixture over a period of three months.

RESULTS AND DISCUSSION

Optical purity and stability of R(+)-phenylethylisocyanate

The commercial supply of $R(+)$-PEIC had a claimed purity of $>98\%$ by GC analysis. Contamination of $R(+)$-PEIC with the $S(-)$-isomer would lead to the formation of four diastereomeric derivatives of propranolol, thereby confounding the chromatographic separation. To check for enantiomeric purity of $R(+)$-PEIC, the reagent was reacted with optically pure propranolol enantiomer and the product was analyzed by HPLC. When $S(-)$- or $R(+)$-propranolol were reacted individually with $R(+)$-PEIC, only a single peak was observed upon chromatography of the product. Taking into account the detection limit of the diastereomeric derivatives, an optical purity of at least 99% was estimated. Since PEIC is sensitive to oxidation after exposure to air, the reagent was stored under nitrogen at refrigeration temperature. For each run, a fresh working solution of $R(+)$-PEIC in diethyl ether was prepared.

Preliminary results using $S(-)$-PEIC from the same supplier indicated the presence of unknown contaminants, as revealed by extraneous late-eluting peaks in the chromatograms of blank reaction products. Therefore the $R(+)$-isomer of PEIC was chosen for use.

Choice of internal standard

Several related aryloxypropanolamine β-blockers were screened for use as internal standard. $(\pm)$-Metoprolol appeared to be a good choice, since it exhibited the most comparable extraction and derivatization characteristics as propranolol. Unfortunately, blank rat serum extracts contained early-eluting peaks that interfered with the diastereomers formed from $(\pm)$-metoprolol. Gal et al. [21] recently reported the use of CDP as an internal standard for the reversed-phase liquid chromatographic assay of $(\pm)$-propranolol. Reaction of racemic CDP with $R(+)$-PEIC produced two well resolved diastereomeric peaks, which eluted after the diastereomeric products of propranolol. Therefore, CDP was chosen as our internal standard.

Optimization of derivatization reaction

The reaction rate of propranolol enantiomers and (±)-CDP with *R*(+)-PEIC was investigated. An 8-ng amount of racemic propranolol and 10 ng of (±)-CDP dissolved in diethyl ether were reacted with 50 μl of an ethereal solution of *R*(+)-PEIC (5 μl/ml) at room temperature. The reaction rate was monitored by measuring the appearance of the diastereomeric derivative peaks at 5, 15, 30, 45 and 60 min after addition of the chiral reagent. Maximum amounts of all four derivatives were formed within 15 min (Table I). *S*/*R*-enantiomer derivative peak-height ratios were near unity at all times during the 60 min incubation, indicating that the two propranolol stereoisomers reacted with *R*(+)-PEIC at the same rate. Constant peak-height ratios were also observed between the propranolol enantiomers and either one of the CDP diastereomeric peaks after 15 min. Hence, the reaction characteristics of the racemic internal standard are sufficiently similar to the analytes.

The extent of derivatization as a function of the amount of isocyanate reagent was investigated. Extracts from 100 μl of serum spiked with a relatively high concentration of racemic propranolol (i.e., 200 ng/ml) were derivatized with varying amounts of *R*(+)-PEIC, ranging from equimolar concentration to as much as 8000-fold molar excess. As shown in Fig. 2, increasing amounts of diastereomers were formed until approximately a 1000- to 2000-fold excess of *R*(+)-PEIC was reached.

In all the experiments described, maximal reaction was accompanied by complete disappearance of the propranolol or CDP peaks. Separate reactions with individual enantiomers of propranolol produced in each case a single diastereomeric product as suggested by the absence of any extraneous peaks other than the diastereomeric peak of interest in the chromatograms. Using NMR spectroscopy, Thompson et al. [17] had previously shown that only the urea derivative of propranolol is formed. Hence, it appeared that the derivatization reactions were rapid and quantitative. Finally, the diastereomeric derivatives remained stable in the reconstitution medium for at least 24–48 h.

TABLE I

YIELD OF DIASTEREOMERIC DERIVATIVES OF PROPRANOLOL ENANTIOMERS AND INTERNAL STANDARD AS A FUNCTION OF REACTION TIME WITH *R*(+)-PEIC

Mean ± standard deviation of fluorescence detector response.

Reaction time (min)	Absolute response (cm)			Response ratio		
	S(−)	*R*(+)	I.S.[a]	*S*(−)/*R*(+)	*S*(−)/I.S.	*R*(+)/I.S.
5	12.9 ± 1.4	13.3 ± 1.5	13.6 ± 2.3	0.97 ± 0.04	0.95 ± 0.10	0.99 ± 0.14
15	15.2 ± 0.9	15.4 ± 0.7	14.7 ± 2.2	0.98 ± 0.01	1.05 ± 0.14	1.06 ± 0.15
30	15.5 ± 1.4	15.8 ± 1.2	14.5 ± 1.8	0.98 ± 0.01	1.07 ± 0.07	1.08 ± 0.05
60	15.4 ± 1.1	15.8 ± 1.1	13.2 ± 0.6	0.99 ± 0.01	1.18 ± 0.07	1.20 + 0.07

[a]The internal standard (I.S.), racemic CDP, yielded two diastereomeric peaks. The early-eluting peak, which most likely represent the product from the (−)-isomer, was used for the internal standard response measurement.

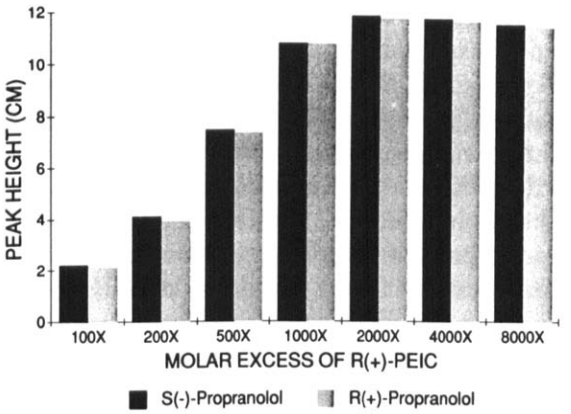

Fig. 2. Yield of diastereomeric derivatives of $S(-)$-propranolol and $R(+)$-propranolol as a function of the molar amount of chiral derivatizing reagent. The amount of diastereomeric products formed was measured by the fluorescence detector response expressed in absolute peak height units.

During the early phase of assay development, notable sample-to-sample variability in peak-height response was observed when the ambient temperature exceeded 25°C. Part of the problem may be related to the volatility of PEIC in diethyl ether. To minimize volatility, the incubation was carried out in the refrigerator (~ 4°C). As can be seen in Table I, reproducible derivatization was achieved as indicated by the low inter-sample coefficient of variation in absolute peak heights and peak-height ratios (i.e., $<10\%$ and 5%, respectively).

The choice of solvent medium for the reaction was also examined. Five organic solvents were investigated: methylene chloride, chloroform (with amylene or ethanol as a preservative), acetonitrile, ethyl acetate, and diethyl ether. Erratic reactions were observed in chloroform preserved with amylene. The reason for the inconsistency is not known. A reproducible and quantitative reaction was observed in the other solvents. Diethyl ether was chosen because of its ease in evaporation.

Extraction recovery

The extraction yield of propranolol enantiomers and the internal standard were determined by comparing peak heights of diastereomeric urea derivatives from serum extracts with those from directly derivatized solutions of racemic propranolol. The recovery of $R(+)$- and $S(-)$-propranolol after extraction from rat serum was 74 ± 3.8 and $78 \pm 3.7\%$, respectively, at a concentration of 25 ng/ml. The recovery of the internal standard CDP from serum was $85 \pm 3.9\%$ when spiked at an amount of 6 ng. The less than complete recovery was probably due to an unavoidable absorptive loss to glassware at the rather low levels of these lipophilic amine compounds.

Optimization of chromatographic conditions

Reversed-phase liquid chromatography was used to separate the diastereomers formed after reaction of propranolol with the chiral reagent PEIC. Thompson et al. [17] employed a C_{18} column for the separation of the diastereomers. The elu-

tion times were in the order of 20 min at a relatively high mobile phase flow-rate of 3.2 ml/min. To reduce column back-pressure and still maintain a reasonable retention time (< 20 min), we elected to use a C_8 column which has a much lower capacity factor for the diastereomeric analytes. A better resolution of the diastereomers was also achieved due to improved peak symmetry.

During the early part of the assay development, a gradual build up of column pressure was observed after repeated sample injection, possibly due to an accumulation of excess $R(+)$-PEIC or by-products of the derivatization. To circumvent this problem, methylene chloride was added to the mobile phase at the concentration of 1.0% to effect a continual clean-up of the column. Also, any residual $R(+)$-PEIC reagent in the derivatized extracts was hydrolyzed by reconstitution in an aqueous medium (i.e., mobile phase) and waiting 20 min before injection of the samples.

The chromatographic conditions were selected to give the highest selectivity for the diastereomers with a minimum resolution factor of 1.33. Under the chosen conditions, the absolute retention times for the diastereomeric derivatives of $S(-)$- and $R(+)$-enantiomers were 13.7 and 14.9 min, respectively. The diastereomeric products of the racemic internal standard, CDP, also eluted as two resolved peaks with retention times of 19.3 and 21.2 min. Fig. 3 shows representative chromatograms of extracts from sera collected from rats before and after intravenous administration of racemic propranolol. Chromatograms from extracts of blank sera from either human subjects or rats were free of interfering peaks. Turbid samples were noted to yield a noisy chromatographic baseline. The problem was easily eliminated by centrifugation at 10 000 g for 2 min to clarify the serum.

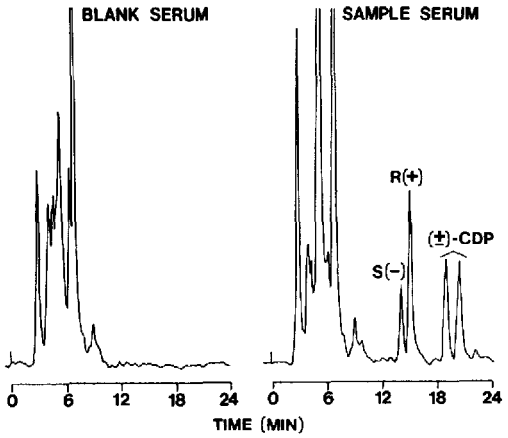

Fig. 3. Representative chromatograms of the diastereomeric derivatives of $S(-)$- and $R(+)$-propranolol and the internal standard, (±)-N-cyclopentyldesisopropylpropranolol [(±)-CDP], in extracts of sera obtained from rats before and after racemic drug administration. The concentration of $S(-)$- and $R(+)$-propranolol in the sample shown were 15.0 and 40.0 ng/ml, respectively. The amount of racemic internal standard added to the sample was 6 ng.

Assay performance

The fluorescence response in terms of peak-height ratio was linear over an enantiomer concentration of 3–48 ng/ml. Minimum assay limit, defined as the sample concentration that provides a detector response with a signal-to-noise ratio of at least 3:1, was determined to be 2–3 ng/ml with a sample volume of 100 μl. A lower limit of sensitivity can be achieved by doubling the volume of the serum specimen.

The within-batch and inter-day reproducibility of the assay were assessed by replicate analysis of rat serum spiked with low and high concentrations of the racemic compound. The within-batch coefficient of variation was 4.5% ($n=5$) for $S(-)$-propranolol and 4.6% ($n=5$) for $R(+)$-propranolol, at a serum enantiomer concentration of 52.5 ng/ml. At the low concentration of 6.33 ng/ml, the respective variability was 6.8% ($n=5$) and 8.1% ($n=5$) for the $S(-)$- and $R(+)$-isomer. For the $S(-)$-enantiomer, the inter-day coefficient of variability over a three-month period was 5.7% ($n=7$) at a concentration of 25.4 ng/ml and 4.5% ($n=7$) at 50.7 ng/ml. For $R(+)$-propranolol, the coefficients of variability were 6.4 and 3.6% at the same respective concentrations.

Assessment of assay interference by propranolol metabolites

Potential interference from the major metabolites of propranolol was investigated. The metabolites examined included the major regioisomers of monohydroxylation (2′-, 4′-, 5′-, and 7′-hydroxypropranolol) and the products of the N-dealkylation pathway (N-desisopropylpropranolol, α-naphthoxylactic acid, α-naphthoxyacetic acid, and propranolol glycol). The metabolites were added to serum and extracted in the same manner as described for propranolol. As expected, little if any of the acidic metabolites were present in the ether extract. Moreover, the chromatographic system is capable of separating the diastereomeric derivatives of propranolol enantiomers from all of the known metabolites and their corresponding products of reactions with $R(+)$-PEIC.

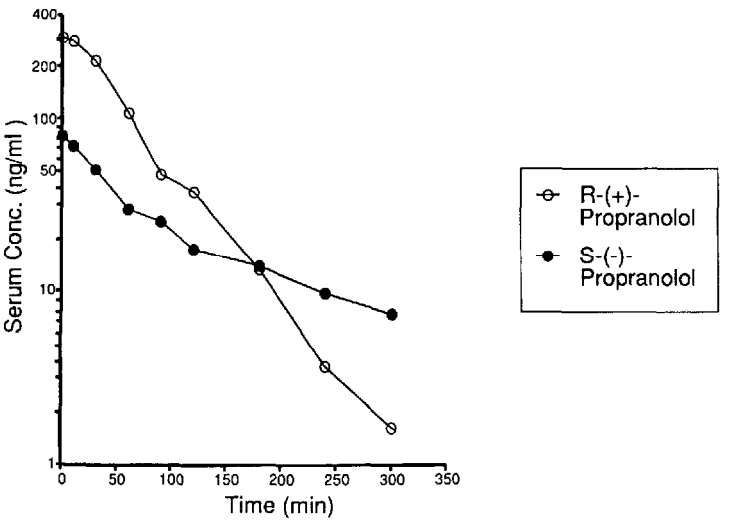

Fig. 4. Typical serum concentration–time course of propranolol enantiomers following a 1.5 mg/kg intravenous dose of the racemic drug in the rat.

Application of the method

The method has been applied successfully to the analysis of several hundred specimens from pharmacokinetic studies with racemic propranolol in chronic renal failure patients and rats with experimentally induced uremia. A typical example of the pharmacokinetic profile of propranolol enantiomers in the rat is presented in Fig. 4. As can be seen, the assay is capable of handling samples containing widely different concentrations of the enantiomers [i.e., $R(+)$- to $S(-)$-propranolol concentration ratios ranging from about 5:1 to 1:5]. Because of the simple preparation procedure, a large number of samples can be processed in a single batch, thereby offering excellent consistency and high throughput.

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