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QUANTIFICATION OF PROPRANOLOL ENANTIOMERS IN SMALL BLOOD SAMPLES FROM RATS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER CHIRAL DERIVATIZATION

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SUMMARY

A high-performance liquid chromatographic (HPLC) technique is described for quantification of $R(+)$- and $S(-)$-propranolol from 100-μl rat blood samples The procedure involves chiral derivatization with *tert.*-butoxycarbonyl-L-leucine anhydride to form diastereomeric propranolol-L-leucine derivatives which are separated on a reversed-phase HPLC column. The method as previously reported has been modified for assaying serial blood microsamples obtained from the rat for pharmacokinetic studies. An internal standard, cyclopentyldesisopropylpropranolol, has been incorporated into the assay and several derivatization parameters have been altered. Standard curves for both enantiomers were linear over a 60-fold concentration range in 100-μl samples of whole rat blood (12 5–750 ng/ml; $r=0.9992$ for each enantiomer). Inter- and intra-assay variability was less than 12% for each enantiomer at 25 ng/ml. No enantiomeric interference or racemization was observed as a result of the derivatization. No analytical interference was noted from endogenous components in rat blood samples. Preliminary data from two male Sprague–Dawley rats given a 2.0 mg/kg intravenous dose of racemic propranolol revealed differential disposition of the two enantiomers. $R(+)$-Propranolol achieved higher initial concentration but was eliminated more rapidly than $S(-)$-propranolol. Terminal half-lives of $R(+)$- and $S(-)$-propranolol were 19.23 and 51.95 min, respectively, in one rat, and 14 50 and 52.07 min, respectively, in the other.

INTRODUCTION

Various methods have been described for the separation and quantification of the enantiomers of propranolol and other β-blockers from biological fluids. Stable isotope-labeled pseudoracemates analyzed by gas chromatography–mass spectrometry (GC–MS) have been employed for plasma and urine samples from both man and dog [1–5]. High-performance liquid chromatographic (HPLC) approaches have also been utilized successfully. Generally, these techniques re-

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quire derivatization with a chiral reagent followed by separation of the resulting diastereomers on reversed-phase [6–13] or normal-phase [14] columns.

Another method has been described utilizing phosgene, an achiral compound, as the derivatizing reagent. The oxazolidone derivatives formed are subsequently separated on a chiral HPLC column [15]. The direct separation of propranolol enantiomers has been achieved on a chiral stationary phase as well [16]. These assays have allowed researchers to accurately characterize the stereoselective pharmacokinetics of some β-blockers, particularly propranolol, in man and in the dog.

However, the use of the rat as a model for investigating the stereoselective disposition of these drugs has hitherto been minimal. Presumably this can be attributed at least in part to the absence of a reliable assay for quantification of the enantiomers of these racemic drugs in small blood samples. In one investigation, propranolol enantiomers were derivatized with a chiral reagent and analyzed by gas chromatography [17]. This method was used to analyze rat blood samples, but large blood volumes (0.5–2.0 ml) acquired by serial sacrifice were needed. In another study, antisera were generated against *l*-propranolol and *dl*-propranolol for development of a stereoselective radioimmunoassay for propranolol isomers in the rat [18]. This assay was also applied to a study in mice [19]. Unfortunately, initial enthusiasm for the technique has been tempered by concerns over its specificity and availability.

The present investigation was initiated to develop an analytical methodology for quantifying propranolol isomers in rat blood microsamples. To achieve this, the chiral derivatization technique initially described by Hermansson [10] was modified and adapted. This approach requires the formation of L-leucine diastereomers of propranolol using *tert.* butoxycarbonyl-L-leucine anhydride as the derivatizing reagent. An internal standard, cyclopentyldesisopropylpropranolol (CDP), was incorported into the assay to enhance its accuracy during the scaling-down process. Minor adjustments in several phases of the extraction and derivatization procedure have been imposed and the assay was applied to a study of the stereoselective disposition of propranolol in male Sprague–Dawley rats.

EXPERIMENTAL

Chemicals

Racemic propranolol hydrochloride was kindly provided by Ayerst Labs. (New York, NY, U.S.A.). *R*(+)- and *S*(−)-propranolol hydrochloride were generously supplied by Dr. Wendel Nelson (College of Pharmacy, University of Washington, Seattle, WA, U.S.A.). CDP was obtained from Pierce (Rockford, IL, U.S.A.) N-*tert.*-Butoxycarbonyl-L-leucine (Boc-L-Leu), N-*tert.*-butoxycarbonyl-D-leucine (Boc-D-Leu) and ammonium phosphate, monobasic were obtained from Sigma (St. Louis, MO, U.S.A.). 1,3-Dicyclohexylcarbodiimide (DCCI), trifluoroacetic acid (TFA), and triethylamine (TEA, Gold label) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Methanol (HPLC grade), methylene chloride (HPLC grade), sodium carbonate, and diethyl ether (reagent grade) were procured from Fisher Scientific (Fairlawn, NJ, U.S.A.). All chemicals and solvents were used

without further purification. Doubly distilled water was used in all aqueous solutions.

Apparatus and chromatographic conditions

The liquid chromatographic system was composed of a Beckman Model 114M solvent delivery system (Beckman Instruments, Fullerton, CA, U.S.A.) and a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.). Detection was carried out with a Perkin-Elmer LS-1 filter fluorimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) with a 228-nm excitation filter and a 290-nm cutoff filter. An Altex Ultrasphere-ODS column (250×2.0 mm, 5 μm particle size; Altex Scientific, Berkeley, CA, U.S.A.) was used for the analysis and the system was equipped with a Brownlee Spheri-5-RP-18 guard column (30 mm×2.1 mm, 5 μm particle size; Brownlee Labs, Santa Clara, CA, U.S.A.). The chromatographic column was maintained at ambient temperature and equilibrated with the mobile phase. The mobile phase consisted of methanol–water containing 0.02 *M* $NH_4H_2PO_4$, pH 2.8 (72:28, v/v). The flow-rate was 0.2 ml/min. The UV scans of underivatized and the derivatized enantiomers of propranolol were performed with a Hewlett Packard 1099 liquid chromatograph with a diode array detector (Waldbronn, F.R.G.).

Extraction and derivatization procedure

Synthesis of the reagent. The derivatization reagent (the anhydride of Boc-L-Leu) was prepared fresh for each use as follows: 2 ml of a 0.25 *M* solution of DCCI in methylene chloride (0.5 mmol) were added to 1.0 mmol of Boc-L-Leu in 3.0 ml of methylene chloride. The mixture was vortexed briefly and allowed to react at 0°C for 75 min. The insoluble urea by-product was filtered off and the reagent solution (supernatant) was maintained at 0°C at all times prior to its use.

Extraction and derivatization. To each culture tube containing 100 μl of whole rat blood and 75 ng of CDP were added 6.0 ml of diethyl ether and 2.0 ml of sodium carbonate (1.0 *M*, pH 9.9). The tubes were agitated for 25 min in a horizontal shaker and centrifuged at 2000 *g* for 10 min. The ether layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen with mild heating (30°C).

The residue was reconstituted in a 250- μl solution of TEA in methylene chloride (300 μmol TEA per 250 μl of solution), followed immediately by the addition of 200 μl of the reagent solution. After briefly vortexing, the tubes were capped and allowed to stand at ambient temperature for 60 min. The derivatization mixture was evaporated at 30°C under a stream of nitrogen. After 17 min, the tubes were removed from the evaporator and 2.0 ml of a 0.1 *M* sodium hydroxide solution were added. The tubes were agitated for 5 min and 6.0 ml of diethyl ether were added. The solutions were then shaken for 15 min and centrifuged at 2000 *g*. The ether layer was collected in a fresh tube and evaporated to dryness at 30°C under nitrogen.

The tubes were subsequently chilled to 0°C in an ice-bath and the residue was reconstituted with 250 μl of TFA. The tubes were vortexed briefly, capped, and returned to the ice-bath. After 7 min, 2.0 ml of 2 *M* sodium hydroxide were added,

followed immediately by 6.0 ml of diethyl ether. The mixtures were shaken and centrifuged and the ether layer was isolated. The derivatized propranolol and internal standard were then extracted into 150 μl of 0.1 *M* orthophosphoric acid. A 20-μl aliquot of the aqueous phase was injected into the HPLC system.

Standard curves

Stock solutions of racemic propranolol base were prepared in methanol at concentrations of 25–1500 ng/ml. The internal standard solution was prepared by dissolving CDP base in methanol to achieve a final concentration of 500 ng/ml. The propranolol stock solutions (100 μl) were then added to successive tubes with 150 μl of the CDP solution, and the methanol was evaporated. Blank whole rat blood (100 μl) was then added to each tube, achieving a CDP concentration of 750 mg/ml and final concentrations of racemic propranolol ranging from 25 to 1500 ng/ml. Standard solutions were extracted and derivatized as outlined above. Standard curves were plotted as peak height ratio (PHR = derivatized propranolol enantiomer peak height/first derivatized CDP enantiomer peak height) versus sample concentration.

Animal study

To evaluate the utility of this assay for animal studies, the disposition of racemic propranolol was examined in two male Sprague–Dawley rats (481 and 477 g). Under diethyl ether anesthesia, cannulae were inserted into the right external jugular and left femoral veins and exteriorized subcutaneously through the back of the neck. After allowing the animals to recuperate overnight, a 2.0 mg/kg bolus dose of propranolol hydrochloride was administered through the femoral cannula followed by 0.6 ml of normal saline. Blood samples (220 μl each) were drawn via the jugular cannula using a previously described microsampling technique [20]. Two 100-μl aliquots from each sample were placed in culture tubes previously spiked with 75 ng of CDP and frozen for later duplicate analysis of *R*(+)- and *S*(−)-propranolol. Samples were collected at 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 180, and 240 min after dosing. At later times (120–240 min) 200-μl aliquots were employed and the concentrations obtained were halved.

RESULTS AND DISCUSSION

The present investigation represents a useful adaptation of the stereoselective propranolol assay initially described by Hermansson [10]. In the orginal study, 1.0-ml samples of human plasma were analyzed against standard curves constructed by plotting peak height versus sample concentration. Unfortunately, considerable variability was encountered when this method of quantification was employed in the current study. This may be attributable in part to the absence of an internal standard and the use of smaller samples of whole blood rather than plasma. The incorporation of an internal standard into the assay was therefore deemed necessary.

CDP possesses several characteristics, all related to its structural similarity to propranolol, which render it the most prudent choice as an internal standard. Differing from propranolol only in the N-alkyl moiety, it is not surprizing to find

that CDP behaves similarly to the drug in various respects. Significant variability in recovery of the propranolol Boc-L-Leu diastereomers was encountered after the numerous extractions, pH adjustments, and chemical manipulations (most notably derivatization) required by this assay. Theoretically, parallel variability in the recovery of the CDP final product will also occur. It is also expected that there is a close similarity in the chemical behavior of the like isomers of the two compounds. Hence, the use of calibration curves based on peak-height ratios (propranolol/CDP) afforded a considerably more reproducible assay in small blood samples than the use of peak heights of derivatized propranolol enantiomers. Moreover, the similarity in fluorimetric properties of CDP and propranolol allowed detection at more optimal excitation and emission wavelengths, enhancing the sensitivity of the assay. CDP's merits as an internal standard in non-stereoselective propranolol assays have been described previously by others [21].

The inclusion of another derivatizable substance into the analytical scheme has necessitated minor adjustments of several parameters in the derivatization procedure. Through a set of short experiments it was found that a reaction time of 60 min was sufficient to completely derivatize all the enantiomers of interest (Fig. 1). Also, whereas Hermansson [10] utilized 100 μl of the derivatizing reagent and 90 μmol of TEA in the derivatization reaction, it was found that 200 μl of the Boc-L-Leu reagent and 300 μmol of TEA worked better in this assay. It was also observed that a critically timed 7-min treatment with TFA at 0°C provided better results than the 5-min treatment with this acid as previously reported. It must further be noted that the most optimal results were obtained when freshly prepared derivatizing reagent was used an when the timing of several crucial phases (e.g. TFA treatment, derivatization reaction time, evaporation of derivatization solution) were carefully controlled. These conditions fostered a degree of derivatization and recovery in excess of 90%.

A sample chromatogram is provided in Fig. 2. Baseline resolution of the derivatized enantiomers of both propranolol and CDP was achieved with the C_{18} microbore column and a mobile phase of methanol – ammonium phosphate buffer

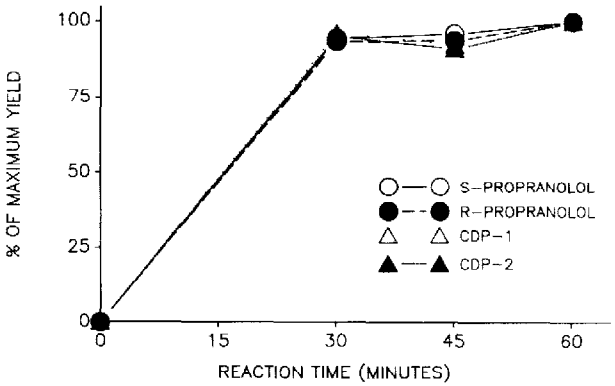

Fig. 1. Formation of Boc-L-Leu derivatives of $R(+)$- and $S(-)$-propranolol, CDP-1 and CDP-2 as a function of derivatization reaction time.

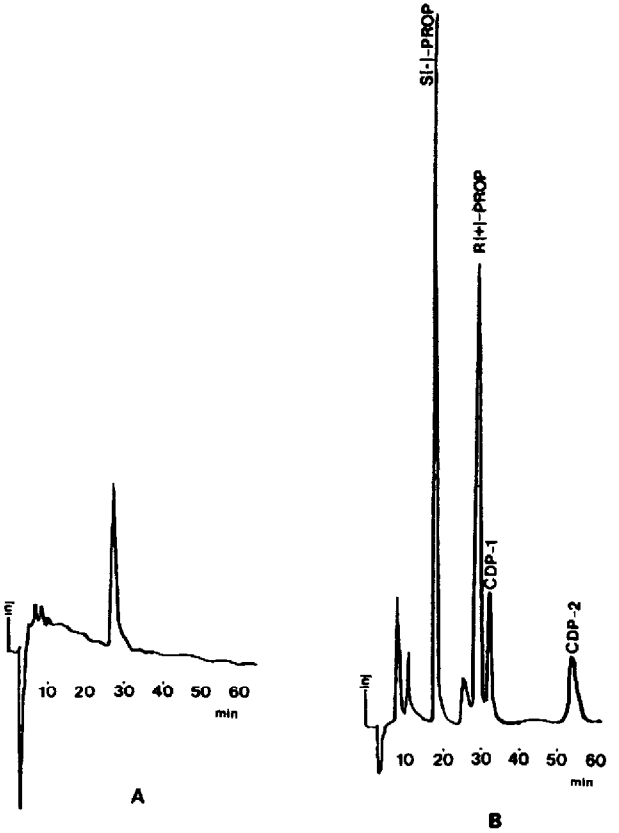

Fig. 2. Chromatograms of (A) a blank rat blood sample and (B) a 100-μl blood sample containing internal standard (CDP) and 500 ng/ml of each propranolol enantiomer (CDP-1 is the first eluting CDP-L-Leu derivative and CDP-2 is the second eluting CDP-L-Leu derivative).

(0.02 *M*, pH 2.8) (72:28). Shorter retention times with comparable enantiomeric resolution have been achieved using a higher percentage of methanol in the mobile phase. Unfortunately, such an alteration also caused the derivatized *R*(+)-propranolol to co-elute with the unidentified peak at 26 min (Fig. 2B). Furthermore, complete separation of these two peaks requires a mobile phase composition that extends the retention time of the derivatized second peak of CDP to 90 min. Thus, a compromise was reached that maximized the separation of the two overlapping peaks and minimized the total time of a sample run. While the *R*(+)-propranolol derivative still elutes on the down slope of the unidentified peak, no discernable impairment in accuracy or sensitivity in its quantification has been observed (Table I).

The nature of the peak at 26 min remains unkown. Presumably, it is a by-product of the derivatization and/or deprotection reactions as its presence has been observed when the derivatization and extraction procedure was performed on blank buffer (i.e. no drug or internal standard present). No interference from endogenous rat blood products was noted (Fig. 2A) and chromatograms in general were very clean (an important consideration for avoidance of peak co-elution).

TABLE I

REPRODUCIBILITY OF THE ASSAY FOR $S(-)$- AND $R(+)$-PROPRANOLOL FROM 100 μl RAT BLOOD SAMPLES

Intra- and inter-day coefficients of variation (C.V) are calculated for each enantiomer.

Expected concentration (μg/ml)	Total number of determinations	Measured concentration (mean ± S.D) (μg/ml)	C.V. (%)
Intra-day S(−)-propranolol			
0.025	9	0.027 ± 0.0015	5.74
0.100	9	0.101 ± 0.0063	6.24
0.400	9	0.423 ± 0.0180	4.26
0.750	8	0.728 ± 0.0360	4.95
Intra-day: R(+)-propranolol			
0.025	9	0.026 ± 0.0024	9 23
0.100	9	0.094 ± 0.0056	5.96
0.400	9	0.414 ± 0.0260	6.28
0.750	8	0.759 ± 0 0064	8.43
Inter-day: S(−)-propranolol			
0.025	9	0.025 ± 0.0028	11.42
0.100	9	0.094 ± 0.0037	3.94
0.400	9	0.399 ± 0.0220	5.51
0.750	9	0.762 ± 0.0380	4.99
Inter-day R(+)-propranolol			
0.025	9	0.026 ± 0.0026	10.00
0.100	9	0.101 ± 0.0030	2.97
0.400	9	0.406 ± 0.0260	6 40
0.750	9	0.743 ± 0.0320	4.31

Standard curves constructed for each enantiomer from the racemic mixture were linear with strong positive correlations over a 60-fold concentration range (12.5–750 ng/ml; $r = 0.9992$ for each enantiomer). Peak-height ratios were used in constructing the calibration curves and were based on peak heights of the derivatized propranolol isomers and the first CDP derivative. The peak corresponding to the first derivatized CDP isomer was at all times narrower and more intense than the second, allowing for more reproducible quantification when employing this peak.

Derivatization and extraction were performed on the isolated enantiomers of propranolol from 100 μl of rat blood. This procedure allowed the identification of the derivatized propranolol isomer peaks. The $S(-)$-propranolol derivative eluted with a retention time of 18 min and the $R(+)$-propranolol derivative eluted at 29 min (Fig. 3). As only trace amounts of the opposite antipode were observed when a single enantiomer was derivatized, it was concluded that no significant racemization occurred during the derivatization. As further verification of the peak identities, the isolated enantiomers of propranolol and CDP were also de-

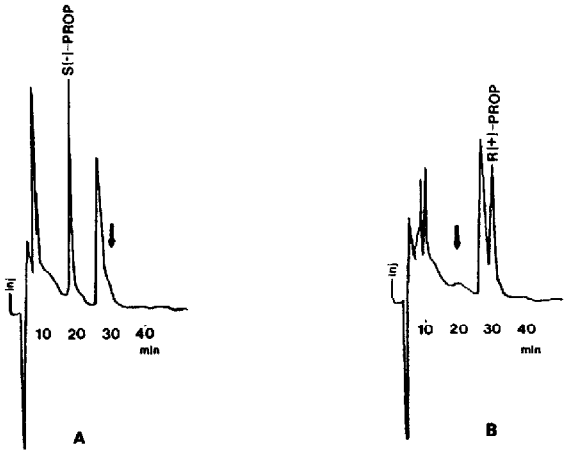

Fig. 3. Racemization test. Chromatograms of 100-μl samples containing (A) 100 ng/ml $S(-)$-propranolol (arrow indicates retention time of $R(+)$-propranolol-L-Leu derivative) and (B) 100 ng/ml $R(+)$-propranolol (arrow indicates retention time of $S(-)$-propranolol-L-Leu derivative).

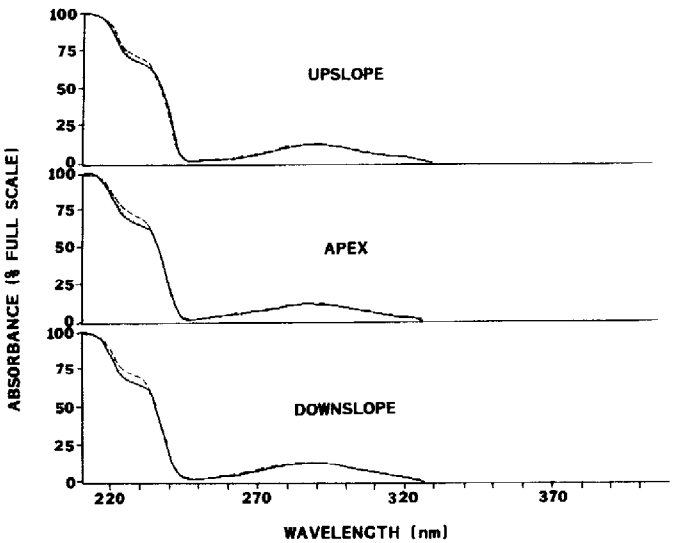

Fig. 4. Photodiode array scans of $S(-)$-propranolol-L-Leu (——), $R(+)$-propranolol-L-Leu (· · ·) and underivatized propranolol (----) taken on the upslope, at the apex, and on the downslope of their respective chromatographic peaks.

rivatized with the reagent Boc-D-Leu (absolute configuration R) rather than Boc-L-Leu (absolute configuration S). R-Propranolol-D-Leu (absolute configuration R,R) and S-propranolol-L-Leu (S,S) are non-superimposable mirror images, as are R-propranolol-L-Leu (R,S) and S-propranolol-D-Leu (S,R). Thus, derivatization of R- and S-propranolol with Boc-D-Leu should result in an elution order which is exactly the reverse of that found after derivatization with Boc-L-Leu (i.e. the R-propranolol derivative should elute before the S-propranolol derivative). As expected, the chromatographic profile following derivatization with Boc-

D-Leu was identical to that following derivatization with Boc-L-Leu, but the enantiomeric elution order was reversed with $R(+)$-propranolol-D-Leu eluting first.

In order to further verify the peaks observed on the chromatogram as propranolol-related products, a UV scan was taken of the peaks as they eluted from the HPLC column using a photodiode array detector. The UV absorbance for the derivatized propranolol enantiomers was found to be superimposable with that of underivatized propranolol (Fig. 4). As the primary naphthyl chromophore in propranolol is unaffected by derivatization and produces a rather unique absorption spectrum, it appears clear the peaks identified as propranolol derivatives are the L-Leu derivatives of R- and S-propranolol. Mass spectral data of the propranolol derivatives were presented earlier by Hermansson [10]. That data taken together with the current work supports the identify of the propranolol derivatives described here.

Inter- and intra-assay variability was tested on multiple standard solutions of racemic propranolol at concentrations of 50, 400, 800, and 1500 ng/ml. The relative standard deviations for each enantiomer at all concentrations were less than 12% (Table I). Owing to the smaller sample size, the sensitivity of this assay compares favorably with that noted by Hermansson [10]. The lower limit of the standard curves noted in the original study was 1.0 ng/ml per enantiomer when using a 1.0-ml sample of human plasma. In the current investigation, standard curves were constructed to 12.5 ng/ml per enantiomer using 100-μl blood, samples, corresponding to an absolute amount of only 1.25 ng for each enantiomer of propranolol. In addition, concentrations as low as 2.5 ng/ml per enantiomer have been detected with a signal-to-noise ratio of 3:1 from 200 μl blood samples.

The blood concentration versus time profile of $R(+)$- and $S(-)$-propranolol in the rats administered a 2.0 mg/kg intravenous dose is shown in Figs. 5 and 6. The marked difference in the disposition of the two enantiomers in rats is apparent from these plots. The $R(+)$-enantiomer achieves higher initial concentrations but is eliminated more rapidly than the $S(-)$-enantiomer. Non-linear regression analysis (NONLIN) revealed terminal half-lives of $R(+)$- and $S(-)$-propranolol of 19.23 and 51.95 min, respectively, in the 481-g rat (Fig. 5). Similar values for the 477-g rat were 14.50 and 52.07 min (Fig. 6). Kawashima et al. [18], employing the stereoselective radioimmunoassay, observed almost identical profiles after 1.0 mg/kg intravenous dose in the rat, reporting terminal half-lives of 23.8 and 52.0 min for $R(+)$- and $S(-)$-propranolol, respectively. These results also compare favorably to the study by Caccia et al. [17] in which they reported similar profiles and terminal half-lives of 44 and 64 min for the $R(+)$- and $S(-)$-isomers, respectively.

That the enantiomers of propranolol behave differently in an in vivo rat model points to the need for a stereoselective assay in blood microsamples. The present analytical method should prove useful in evaluating how physiological changes and co-adminstration of drugs in rats influence the stereoselective disposition and pharmacological effects of this widely used drug.

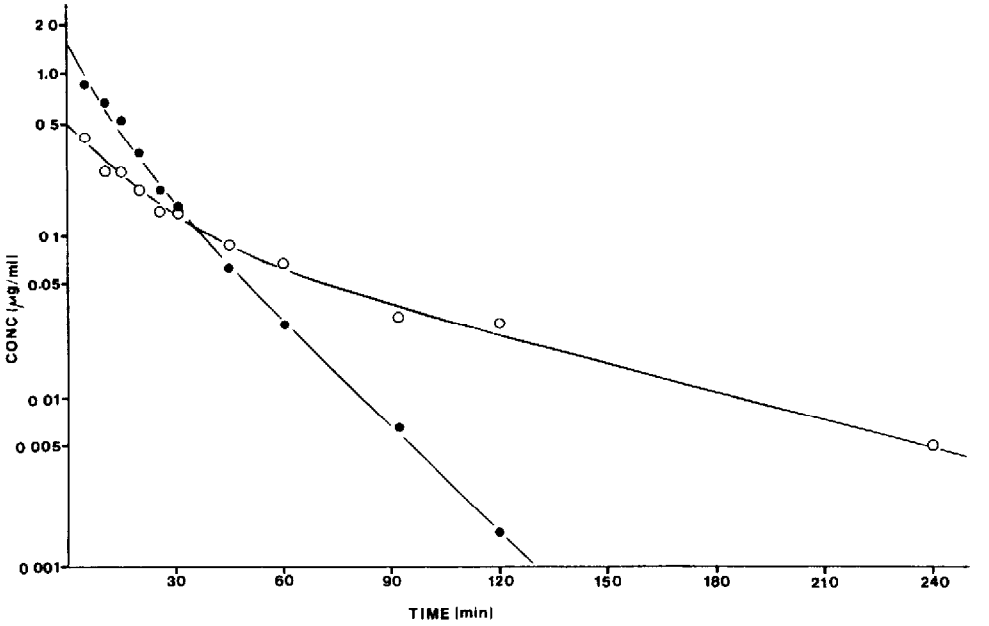

Fig. 5. Log concentration–time profiles for propranolol enantiomers in a 481-g rat after a 2.0 mg/kg intravenous dose of racemic propranolol (●, *R*-isomer, ○, *S*-isomer). Lines represent the best-fit curves determined by non-linear regression analysis (NONLIN).

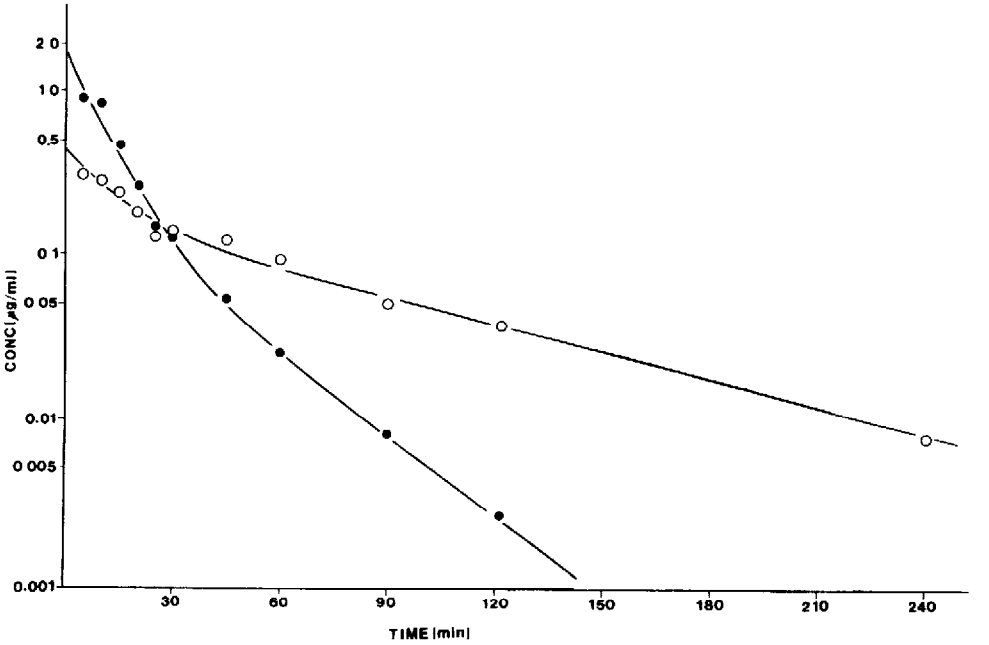

Fig. 6. Log concentration–time profiles for propranolol enantiomers in a 477-g rat after a 2 0 mg/kg intravenous dose of racemic propranolol (●, *R*-isomer, ○, *S*-isomer). Lines represent the best-fit curves determined by non-linear regression analysis (NONLIN).

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