Simultaneous Determination of Propranolol Enantiomers in Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

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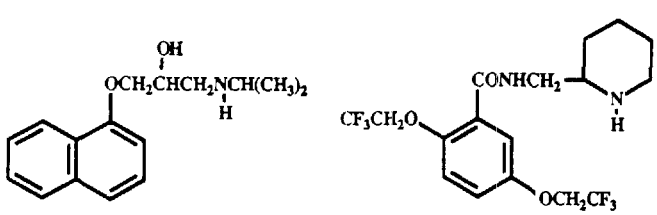
Abstract □ A simple, rapid, and sensitive assay for the simultaneous quantification of the (−)- and (+)-propranolol in human and dog plasma is described using a reversed-phase high-performance liquid chromatography (HPLC) system with fluorescence detection. The method involves extraction of propranolol enantiomers from plasma into 1% 1-butanol in *n*-hexane at basic pH, followed by evaporation of the organic phase and the formation of diastereomeric derivatives with the chiral reagent (−)-menthyl chloroformate. (+)-Flecainide is used as the internal standard. The limiting concentration of each enantiomer that can be detected is 1 ng/mL plasma. In six normal human volunteers, who received a single oral dose of 80 mg of racemic propranolol, the plasma levels of the (−)-enantiomer were always higher than those of the (+)-enantiomer with a mean (−):(+) ratio of 1.38. The half-lives of both the enantiomers were similar (3.5 ± 0.3 h). In six normal male mongrel dogs given a single intraportal dose of 40 mg of racemic propranolol, the plasma levels of the (−)-enantiomer were always lower than those of the (+)-enantiomer with a mean (−):(+) ratio of 0.48. The half-life of the (−)-enantiomer (73.3 ± 16.2 min) was shorter than that of the (+)-enantiomer (87.1 ± 18.1 min).

Propranolol, a β-adrenergic receptor blocking agent, is widely used as the commercially available racemic mixture in the treatment of hypertension, thyrotoxicosis, cardiac arrhythmias, and other diseases. The (−)-enantiomer is 60–100 times more potent as a β-blocker than the (+)-enantiomer and is probably responsible for almost all of the pharmacological effects.[1,2] It is reported that after the administration of racemic propranolol to animals the enantiomers have different disposition kinetics.[3–10] Differences in their disposition may, therefore, be important clinically. However, studies on enantioselective disposition have been limited to examining normal healthy subjects or laboratory animals.[3–10] The present study was prompted by a need to examine the pharmacokinetic basis for enantioselective propranolol disposition in different patient populations. These include different disease states, patients undergoing concurrent therapy with other drugs, and patients with known phenotypic differences in metabolism (such as the debrisoquine poor metabolizers).

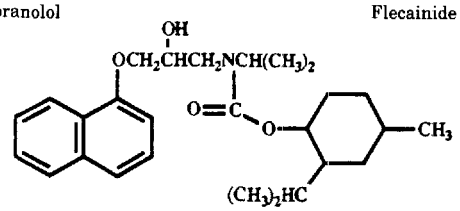
Methods presently available to quantify propranolol enantiomers simultaneously after administration of the racemic mixture include stable isotope-labeled pseudoracemates with analysis by gas chromatography-mass spectrometry (GC-MS)[5,6] and high-performance liquid chromatography (HPLC) after chiral derivatization.[7–14] However, some of these methods require laborious work-up, lack an internal standard in the assay, and/or require large sample volumes (10–20 mL of blood)[9] to obtain a reasonable detection limit. Two methods based on HPLC that have adequate sensitivity for plasma determinations have been developed previously.[7,11] These methods employed *N*-trifluoroacetyl-(−)-prolyl chloride (TPC) as a derivatizing reagent to prepare diastereomers of propranolol that could be separated by HPLC. However, there are several problems with these methods: first, TPC is known to be contaminated by the (+)-enantiomer,[7,11] second, TPC can racemize on storage,[7,15,16] and third, the work-up for the analysis of plasma samples required a number of tedious steps.

In light of the foregoing, it is evident that a simple and sensitive routine assay is required so that quantification of plasma propranolol enantiomers can be carried out in large patient populations. We report a method that involves the preparation of diastereomeric derivatives of propranolol with the chiral reagent, (−)-menthyl chloroformate (MCF). This is followed by separation of the diastereomers on a reversed-phase column. Analysis is carried out with high sensitivity by the use of a fluorescence detector using (+)-flecainide (see structure) as the internal standard. A single extraction of plasma is required. The chiral derivatization reagent (MCF) does not undergo racemization either during storage or on reaction with the plasma extract. The applicability of the procedure has been demonstrated by the analysis of plasma from normal volunteers and laboratory animals (dog). Experimental conditions are discussed for the extraction, derivatization, separation, and quantification of the propranolol enantiomers.

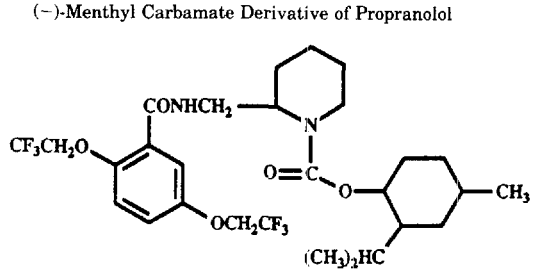


Propranolol

Flecainide



(−)-Menthyl Carbamate Derivative of Propranolol



(−)-Menthyl Carbamate Derivative of Flecainide

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Experimental Section

Chemicals and Reagents—Ultraviolet grade methanol, *n*-hexane, 1-butanol, and acetonitrile were obtained from Burdick and Jackson Laboratories. Triethylamine and (−)-menthyl chloroformate were purchased from Aldrich Chemical. All other chemicals were reagent grade. (±)-Propranolol (Inderal, Ayerst Laboratories, New York, NY) was used throughout this study as its hydrochloride. The (−)- and (+)-propranolol enantiomers and (+)-flecainide were obtained from Imperial Chemical Industries (Macclesfield, Cheshire, Great Britain) and Riker Laboratories (St. Paul, MN), respectively.

Instrumentation—Reversed-phase high-performance liquid chromatography (HPLC) was performed utilizing an M6000 solvent delivery system, a WISP 710B automatic injector (Waters Associates, Milford, MA), and a Spherisorb ODS-2 column (250 × 4.6 mm, 5 μm; Alltech Associates, Deerfield, IL). The column eluant was monitored on a variable-wavelength Schoeffel FS 970-LC fluorometric detector (Schoeffel Instrument, Westwood, NJ) set on an excitation wavelength of 228 nm and an emission cut-off filter of 340 nm. The fluorescence output was recorded on a dual-channel recorder (Linear Instruments, Irvine, CA) and/or on a Data Module (Waters Associates) at speeds of 4 in/h and 0.25 cm/min, respectively.

Fast atom bombardment-mass spectrometry (FAB-MS) was carried out on a VG 70/250 double-focusing magnetic sector instrument at a resolving power of 2000. Samples (5 μg) were analyzed in a 3-nitrobenzyl alcohol matrix on a stainless steel probe tip at ambient temperature using xenon bombardment at ~8 keV. Gas chromatography-mass spectroscopy (GC-MS) was carried out on a Nermag R1010C quadrupole mass spectrometer interfaced with a Varian Vista gas chromatograph. Electron impact (EI) spectra were obtained at 70 eV. Gas chromatography was carried out on a 30 m SPB-5 fused silica column (0.32 mm id; 0.25-μm coating thickness) using helium as the carrier gas at a flow of 1 mL/min. Injections were made in the splitless mode with the injector temperature held at 250 °C. Derivatization for GC-MS analysis was carried out by overnight reaction of the propranolol carbamate derivative (5 μg) and 25 μL of bis(trimethylsilyl) trifluoroacetamide at room temperature.

Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy—Spectra were recorded on an IBM-NR 300 MHz spectrometer in deuterochloroform and are reported in ppm relative to tetramethylsilane as internal standard.

Mobile Phase—The HPLC mobile phase consisted of methanol:water (890:110, v/v). The mixture was deaerated for 40 min using a sonicator and allowed to come to room temperature (25 °C) before being pumped through the column at a rate of 1 mL/min.

Extraction Procedure—To 1 mL of plasma in a 20-mL Teflon-lined, screw-capped, glass, round-bottomed centrifuge tube were added 50 μL (125 ng) of (+)-flecainide solution (2.5 μg/mL), 1 mL of 1 M NaOH, and 8 mL of 1% 1-butanol in *n*-hexane. The mixture was then shaken vigorously for 3 min and centrifuged for 5 min at 1700 rpm. A 7-mL aliquot of the organic phase was transferred to a vial and evaporated to dryness under a stream of nitrogen at 50 ± 2 °C in a waterbath. The residue was then dissolved in a 100-μL volume of 0.4% triethylamine in acetonitrile:methanol (50:50, v/v), and 50 μL of a 0.023 M solution of (−)-MCF in acetonitrile was then added. After mixing thoroughly for 15 min, an aliquot of the reaction mixture was injected onto the HPLC for propranolol enantiomer quantification.

Standards—Stock solutions (100 μg/mL) of racemic propranolol and (+)-flecainide were prepared in methanol and were stored at −20 °C. To obtain a standard calibration curve, aliquots (5, 10, 20, 40, 80, and 160 μL) from a working standard racemic propranolol solution (1 μg/mL) were used to spike 1-mL drug-free plasma samples containing 50 μL of a 2.5 μg/mL solution of (+)-flecainide. This gave a calibration curve ranging from 2.5 and 80 ng/mL for each enantiomer for a sensitivity setting of 1 or 0.5 μA.

Rate and Stability of Propranolol Derivatization with (−)-MCF—To establish the rate and stability of derivatization, drug-free plasma (1 mL) samples were spiked with racemic propranolol in concentrations ranging from 5 to 70 ng/mL plasma. After extraction, evaporation, and treatment with (−)-MCF, the samples were kept at room temperature (25 °C), 4 °C, and/or −20 °C and analyzed periodically for the respective diastereoisomers. The values obtained were compared with those of freshly extracted samples.

Plasma Quality Control—In this study, four sets of drug-free human pooled plasma samples were spiked with increasing concentrations (21.8, 49.8, 99.0, and 147.8 ng/mL plasma) of racemic propranolol solution. After transferring the plasma (1 mL) samples into 20-mL screw-capped centrifuge tubes, they were stored at −20 °C and assayed in triplicate, bi-weekly, for eight weeks to examine intra- and interday variations. The results were compared with those of freshly extracted spiked plasma samples.

Reproducibility—Reproducibility studies for the analysis of the (−)- and (+)-propranolol were performed at five concentrations (2.5, 7.5, 12.5, 17.5, and 25 ng/mL plasma) of each enantiomer, using four samples at each concentration. This was carried out by adding 5, 15, 25, 35, and 50 ng of racemic propranolol standard solution (500 ng/mL) into 1 mL of drug-free plasma. The samples were analyzed against a standard calibration curve according to the procedure described above.

Identification of the Derivatives—The structures of the propranolol derivatives were assigned by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of racemic propranolol menthyl carbamate showed (δ, ppm): 8.18 (m, 1 H, H-8), 7.28 (m, 1 H, H-4), 7.42 (m, 4 H, H-3, H-5, H-6, H-7), 6.83 (d, 1 H, H-2), 4.62 (m, 1 H, C*H*OCON), 4.12 (m, 3 H, CH_2C*H*OH), 4.05 (m, 1 H, NC*H*), 3.48 (m, 2 H, CH_2NH), and 2.06 to 0.80 (m, 23 H). Further confirmation of the structures of the propranolol derivatives was carried out by MS. Individual peaks that eluted from the HPLC column were evaporated and the derivatives were dissolved in methanol prior to analysis by positive ion FAB-MS and GC-MS as described above. The structure of the flecainide derivative was confirmed by FAB-MS.

Pharmacokinetics—*Human Subjects*—Six healthy male volunteers (20–40 years old) each took a single oral dose of 80 mg of racemic propranolol. At timed intervals, venous blood was drawn into heparinized glass tubes and centrifuged, and the 1-mL plasma samples obtained were saved at −20 °C pending analysis of the propranolol enantiomers.

Dogs—Six healthy male mongrel dogs (27 ± 2.4 kg) who had catheters chronically implanted 7 d prior to the study, each received a single intraportal dose of 40 mg of racemic propranolol. At timed intervals, arterial blood was drawn into heparinized glass tubes, and the plasma samples obtained were stored at −20 °C pending analysis of the enantiomers.

The area under the plasma concentration–time curves from time zero to infinity (AUC) was determined according to the log trapezoidal rule after extrapolating the curves to infinity, and the results are expressed as means ± SE. Plasma half-lives were calculated by regression analysis, and the results are expressed as means ± SE.

Results and Discussion

An assay has been developed for the simultaneous determination of the (+)- and (−)-enantiomers of plasma propranolol. The method involves quantification of the diastereomers that are formed by reaction of the individual enantiomers with the chiral reagent (−)-MCF. Chloroformates have been used previously for the conversion of chiral drugs to their respective diastereomers so that they could subsequently be separated by HPLC.[17,18] There are two reports on the use of menthyl chloroformate for the derivatization of secondary amines.[19,20] However, chiral analysis was carried out by GC[19] or column chromatography.[20] Recently, (−)-MCF has been used in our laboratory for the preparation of diastereomeric carbamate derivatives of the antiarrhythmic drug encainide.[21] The reaction of (−)-MCF with racemic propranolol was carried out in 0.4% triethylamine in acetonitrile:methanol (50:50, v/v) at room temperature. The ^{1}H NMR spectrum of the reaction product showed that a single menthyloxy group had been introduced. The chemical shift of the methine hydrogen adjacent to the secondary hydroxyl moiety was identical to that in propranolol itself (δ 4.12). If the menthyloxy group had been attached at this position, then there would have been a shift downfield 1.0 to 1.4 ppm. Further proof of structure was obtained by preparation of the monoacetate of propranolol menthyl carbamate. The methine hydrogen adjacent to the acetoxy moiety was now shifted downfield to 5.52 ppm. This confirmed that derivatization with menthyl chloroformate had occurred on the more nucleophilic nitrogen atom rather than on the hydroxyl

group. A similar observation was made in the derivatization of aminoalcohols with β-naphthylchloroformate.[17]

Analysis of the propranolol menthyl carbamate derivative by reversed-phase HPLC revealed the presence of two peaks with identical areas, at retention times of 12.00 and 13.00 min, respectively. The elution order of the individual diastereomers was established by separate derivatization of each optically pure enantiomer. The diastereomer prepared from the (−)-enantiomer eluted first. Individual propranolol enantiomers were further characterized by positive ion FAB-MS. The (+)-propranolol derivative isolated by HPLC showed an intense $(M+H)^+$ at *m/z* 442, with other characteristic fragment ions at *m/z* 304, 298, 288, 260, and 160 (Figure 1; fragmentations I, II, III, IV, and V). The FAB spectra for (+)- and (−)-propranolol derivatives were identical. The trimethylsilyl ether derivatives of the menthyl carbamate diastereomers from propranolol could not be separated by GC-MS. Their EI mass spectra were identical and in agreement with the assigned structures. The molecular ions appeared at *m/z* 513, and structurally significant fragment ions were observed at *m/z* 370, 360, and 232. The menthyl carbamate derivative of (+)-flecainide showed ions corresponding to intense $(M+Na)^+$ and $(M+H)^+$ at *m/z* 619 and 597, respectively, in its positive FAB-MS spectrum (Figure 2). Other fragment ions at *m/z* 415, 301, and 266 (fragmentations I, II, and III) were consistent with the assigned structures. The proposed structure of the derivative resulting from reaction of propranolol and flecainide with (−)-MCF to form carbamate derivatives are shown above.

Blank plasma extracts yielded no interfering peaks from endogenous substances. No fluctuations were observed in the baseline 6 min after injection and all artifact peaks had eluted by this time (Figure 3A). Efficiency of derivatization was independent of reaction time (5 min to 48 h) and the derivatives were stable at room temperature for at least 48 h. No interfering peaks were observed in the analysis of drug-free plasma to which racemic propranolol and (+)-flecainide internal standard had been added (Figure 3B). Similarly, there were no interfering peaks in plasma derived from humans dosed with racemic propranolol (Figure 3C).

Analysis of multiple human plasma samples for propranolol enantiomers spiked with 5, 15, 25, 35, and 50 ng/mL of racemic propranolol gave excellent reproducibility, linearity, and recovery. Statistical analyses (Table I) by linear regression showed correlation coefficients of 0.99786 and 0.99790, slopes of 0.98834 and 0.977245, and intercepts of 0.23126 and 0.47769 for (−)- and (+)-propranolol, respectively. Intra- and interday variations of the individual enantiomers were also examined over a period of 8 weeks using human plasma quality control samples containing increasing concentrations of racemic propranolol. The results summarized in Table II

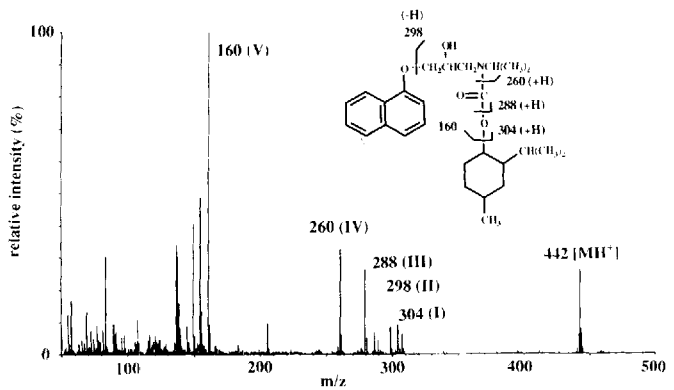

Figure 1—Positive FAB mass spectrum of (−)-menthyl carbamate derivative of (+)-propranolol.

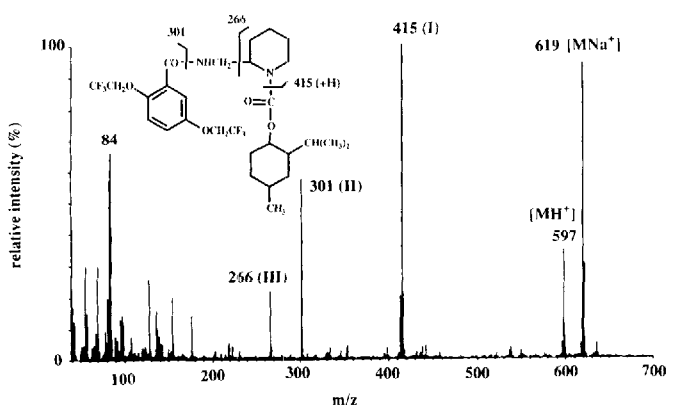

Figure 2—Positive FAB mass spectrum of (−)-menthyl carbamate derivative of (+)-flecainide.

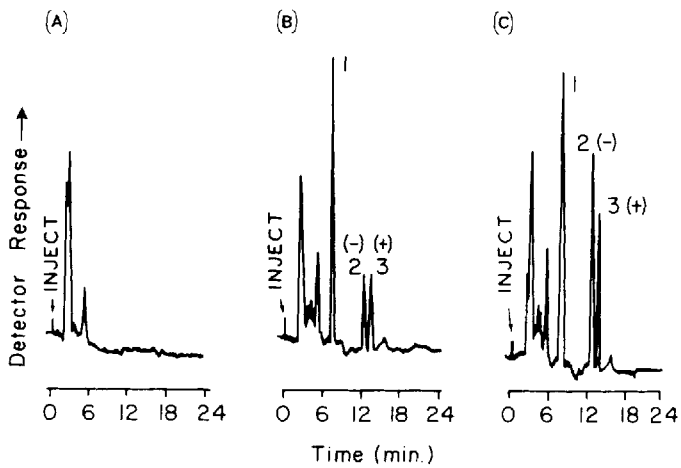

Figure 3—Chromatograms resulting from derivatization with (−)-MCF of the following: (**A**) extract of 1 mL of drug-free human plasma; (**B**) extract of 1 mL of drug-free human plasma spiked with 10 ng of racemic propranolol [peak 1 = 125 ng of the internal standard; (+)-flecainide, peaks 2 and 3 = (−)- and (+)-propranolol, respectively]; (**C**) extract of 1 mL of human plasma 8 h following a single oral dose of racemic propranolol (80 mg) [peak 2 (−)-propranolol = 13.8 ng; peak 3 (+)-propranolol = 9.8 ng]. The instrument conditions were as follows: excitation wavelength = 228 nm; emission cut-off filter = 340 nm; range = 0.5 μA; volume injected = 50 μL; mobile phase = methanol:water (890:110, v/v); flow rate = 1 mL/min.

Table I—Evaluation of the Assay Precision, Linearity, and Accuracy for (−)- and (+)-Propranolol[a]

Spiked (±)-Propranolol Concentration, ng/mL	Sample Concentration (Mean ± SD; n = 6), ng/mL		Coefficient of Variation, %	
	(−)	(+)	(−)	(+)
5	2.5 ± 0.18	2.7 ± 0.16	7.2	5.9
15	7.5 ± 0.19	7.6 ± 0.22	2.5	2.9
25	12.9 ± 0.58	12.9 ± 0.55	4.5	4.3
35	17.7 ± 0.65	17.8 ± 0.62	3.7	3.5
50	24.7 ± 0.65	24.5 ± 0.55	2.6	2.2

[a] Human drug-free plasma (1 mL) samples containing increased concentrations of (±)-propranolol were compared with a standard human plasma calibration curve containing concentrations of (±)-propranolol from 5 to 70 ng; instrument conditions: excitation wavelength = 228 nm; emission cut-off filter = 340 nm; range = 0.5 μA; volume injected = 50 μL; mobile phase = methanol:water (890:110, v/v); flow rate = 1 mL/min.

Table II—Intra- and Interassay Variation of (−)- and (+)-Propranolol in Human Plasma[a]

Spiked (±)-Propranolol Concentration, ng/mL	Intraassay Variation[b] Coefficient of Variation, %		Interassay Variation[c] Sample Concentration Mean ± SD, ng/mL		Interassay Variation[c] Coefficient of Variation, %	
	(−)	(+)	(−)	(+)	(−)	(+)
21.8	2.1–8.0	1.9–8.0	10.2 ± 0.79	10.5 ± 0.66	7.7	6.3
49.8	2.7–6.4	2.1–4.8	24.6 ± 0.75	25.3 ± 0.63	3.0	2.5
99.0	1.5–4.7	2.0–6.3	49.1 ± 1.55	49.7 ± 1.14	3.2	2.3
147.8	1.0–4.9	2.0–4.2	73.1 ± 1.12	73.7 ± 0.74	1.5	1.0

[a] Human plasma (1 mL) quality control samples containing increased concentrations of (±)-propranolol were assayed periodically and compared with a standard human plasma calibration curve containing concentrations of (±)-propranolol from 10 to 160 ng; instrument conditions: excitation wavelength = 228 nm; emission cut-off filter = 340 nm; range = 1 μA; volume injected = 50 μL; mobile phase = methanol:water (890:110, v/v); flow rate = 1 mL/min. [b] Intraday variation was a range over a 8-week period; n = 3. [c] Interday variation was performed over a 8-week period; n = 5.

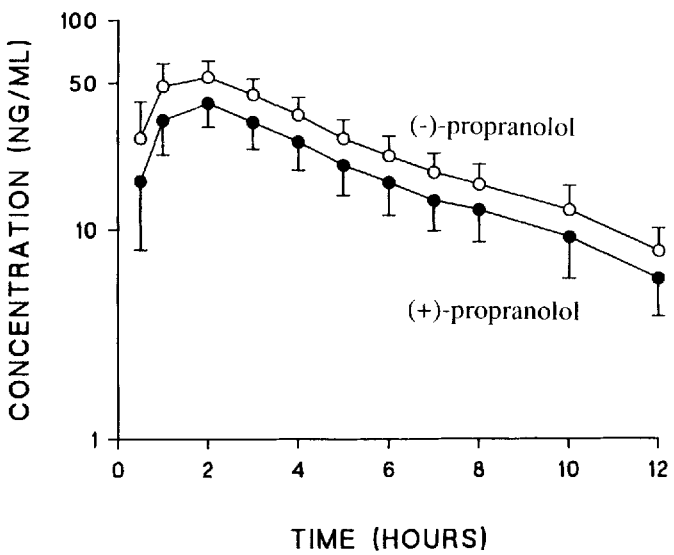

Figure 4—Semilogarithm plot of the plasma concentrations–time profile (mean ± SE) for (−)-propranolol (○) and (+)-propranolol (●) in six subjects following a single oral dose of racemic propranolol (80 mg).

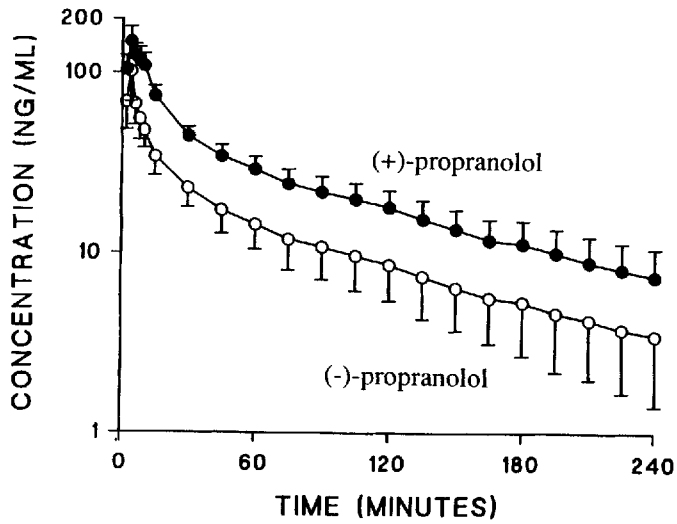

Figure 5—Semilogarithm plot of the plasma concentrations–time profile (mean ± SE) for (−)-propranolol (○) and (+)-propranolol (●) in six dogs following a single intraportal dose of racemic propranolol (40 mg).

show that intra- and interday coefficients of variation ranged between 1 and 8% for both enantiomers. Plasma standard calibration curves for (−)- and (+)-propranolol at concentrations ranging between 5 and 160 ng of racemic propranolol-spiked plasma over a period of 8 weeks gave correlation coefficients of >0.99 for each enantiomer.

The high sensitivity of the present HPLC method is largely the result of using an excitation wavelength of 228 nm and a 340-nm cut-off emission filter. Hermansson and von Bahr[11] used an excitation wavelength of 210 nm and a 340-nm cut-off emission filter. These settings give only 33% of the response obtained with an excitation wavelength of 228 nm. Other investigators use an excitation wavelength of 295 nm, which gives only 7% of the response that is obtained at 228 nm.

The assay is sensitive enough to quantify therapeutic concentrations of propranolol enantiomers in plasma and is suitable for use in pharmacokinetic studies. The plasma concentrations of (−)- and (+)-propranolol measured in six subjects after each received a single oral dose of 80 mg of racemic propranolol show that the concentration of the (−)-propranolol was always higher than that of the (+)-enantiomer (Figure 4), and thus the AUC for the (−)-propranolol was higher (352 ± 76 ng/mL/h) than that for the (+)-enantiomer (250 ± 64 ng/mL/h). The plasma half-lives were similar (3.5 ± 0.3 h) for both enantiomers. Similar findings were reported by Hermansson and von Bahr.[11] The plasma clearance for (−)-propranolol (39.2 ± 10.9 mL/min/kg) was markedly lower than that for (+)-propranolol (60.9 ± 17.9 mL/min/kg). Thus, in normal subjects, plasma propranolol shows enrichment for the active (−)-enantiomer. This confirms the need to rigorously determine whether enantioselective differences in plasma concentrations of the two enantiomers exist in different patient populations.

In the dog, the plasma concentration of (−)-propranolol was always lower than that of the (+)-enantiomer (Figure 5), with subsequent lower AUC. This finding was also reported earlier by Walle and Walle.[5] The plasma half-life for (−)-propranolol (73.4 ± 16.3 min) was lower than that for (+)-propranolol (87.1 ± 18.1 min), with plasma clearances of 10.3 ± 3.6 L/min for the (−)-enantiomer compared with only 3.7 ± 0.8 L/min for the (+)-enantiomer. George et al.[22] reported a longer plasma half-life for the (−)-isomer after administering the enantiomers separately to humans and dogs. It is of interest to note that the stereoselective metabolism differs between humans and dogs so that the (−)-propranolol:(+)-propranolol ratio of the AUCs was 1.38 in humans, but only 0.48 in the dog. This confirms previous reports by Silber and Riegelman.[7]

In conclusion, several HPLC methods for the simultaneous determination of propranolol enantiomers in plasma have been published. The presently described method is a considerable improvement over those reported previously not only because of its selectivity and sensitivity, but also because of its simplicity and rapidity. It is appropriate for routine use in clinical laboratories as long as flecainide is not present in the sample. The standard curve for each enantiomer is linear over

a wide range of 2.5 to 100 ng/mL, with a detection limit (twice baseline) of 1 ng/mL. Only 1 mL or less of plasma is required for measuring the unconjugated enantiomers. However, following enzymatic hydrolysis, the same procedure may be used for measuring total propranolol enantiomers. This method can be applied in pharmacokinetic research studies to examine differences that may exist in bioavailability, disposition, and rate of elimination of the individual enantiomers in humans and animals. It can also be easily employed for therapeutic monitoring of these enantiomers in chronically treated patients who are not receiving concurrent flecainide therapy. The intra- and interday coefficients of variation at different concentrations (Table I and II) illustrate that the assay is accurate and reproducible.

References and Notes

1. Potter, L. T. *J. Pharmacol. Exp. Ther.* **1967**, *155*, 91–100.
2. Barrett, A. M.; Cullum, V. A. *Br. J. Pharmacol.* **1968**, *34*, 43–55.
3. Kawashima, K.; Levy, A.; Spector, S. *J. Pharmacol. Exp. Ther.* **1976**, *196*, 517–523.
4. Caccia, S.; Guiso, G.; Ballabio, M.; DePonte, P. *J. Chromatogr.* **1979**, *172*, 457–462.
5. Walle, T.; Walle, U. K. *Res. Commun. Chem. Path. Pharmacol.* **1979**, *23*, 453–464.
6. Bai, S. A.; Walle, U. K.; Wilson, M. J.; Walle, T. *Drug Metab. Dispos.* **1983**, *11*, 394–395.
7. Silber, B.; Riegelman, S. *J. Pharmacol. Exp. Ther.* **1980**, *215*, 643–646.
8. Silber, B.; Holford, N. H. G.; Riegelman, S. *J. Pharm. Sci.* **1982**, *71*, 699–704.
9. Von Bahr, C.; Hermansson, J.; Tawara, K. *Br. J. Clin. Pharmacol.* **1982**, *14*, 79–82.
10. Von Bahr, C.; Hermansson, J.; Lind, M. *J. Pharmacol. Exp. Ther.* **1982**, *222*, 458–462.
11. Hermansson, J.; Von Bahr, C. *J. Chromatogr.* **1980**, *221*, 109–117.
12. Thompson, J. A.; Holtzman, J. L.; Tsuru, M.; Lerman, C. L.; Holtzman, J. L. *J. Chromatogr.* **1982**, *238*, 470–475.
13. Sedman, A. J.; Gal, J. *J. Chromatogr.* **1983**, *278*, 199–203.
14. Wilson, M. J.; Walle, T. *J. Chromatogr.* **1984**, *310*, 424–430.
15. Gal, J. *J. Pharm. Sci.* **1977**, *66*, 169–172.
16. Manius, G.; Tscherne, R. *J. Chromatogr. Sci.* **1979**, *17*, 322–326.
17. Doyle, T. D.; Adams, W. M.; Fry, F. S.; Wainer, I. W. *J. Liq. Chromatogr.* **1986**, *9*, 455–471.
18. Einarsson, S.; Joseffson, B.; Moller, P.; Sanchez, D. *Anal. Chem.* **1987**, *59*, 1191–1195.
19. Westley, J. W.; Halpern, B. *J. Org. Chem.* **1968**, *33*, 3978–3980.
20. Pirkle, W. H.; Hauske, J. R. *J. Org. Chem.* **1977**, *42*, 2436–2439.
21. Prakash, C.; Jajoo, H. K.; Mayol, R. F.; Blair, I. A., submitted for publication in *J. Chromatogr.*
22. George, C. F.; Fenyvesi, T.; Conolly, M. E.; Dollery, C. T. *Eur. J. Clin. Pharmacol.* **1972**, *4*, 74–76.

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