Combined High-Performance Liquid Chromatographic Procedure for Measuring 4-Hydroxypropranolol and Propranolol in Plasma: Pharmacokinetic Measurements following Conventional and Slow-Release Propranolol Administration

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Abstract □ An assay is described for the simultaneous determination of propranolol and its active metabolite, 4-hydroxypropranolol, in human plasma. Both compounds were separated from an ethereal extract by high-performance liquid chromatography employing a C_{18} bonded-phase column. Detection of the effluent was by fluorescence. Suitable fluorescent spectrometers and wavelength settings that allow optimum detection of both compounds have been described. The limit of sensitivity was 2 ng/ml for both propranolol and 4-hydroxypropranolol. Mean peak plasma levels of propranolol and 4-hydroxypropranolol in six patients receiving a single dose of a slow-release 160-mg formulation of propranolol were 28 and 6 ng/ml, respectively. These levels were about one-tenth the level obtained following a single conventionally prepared dose of propranolol (160 mg). Peak levels were delayed and plasma levels of propranolol persisted for a longer period with the slow-release formulation. Area under the curve estimates suggested that the bioavailability of the slow-release formulation following single-dose administration was about one-third that of the conventional preparation.

Keyphrases □ High-performance liquid chromatography—simultaneous assay of propranolol and 4-hydroxypropranolol, conventional and slow-release formulations compared □ Propranolol—high-performance liquid chromatographic assay with 4-hydroxypropranolol, conventional and slow-release formulations compared □ Pharmacokinetics—propranolol, conventional and slow-release formulations compared □ Bioavailability—propranolol, conventional and slow-release formulations compared

4-Hydroxypropranolol is formed by hepatic microsomal enzymes (1) following oral administration of propranolol (2). This active metabolite possesses β-adrenoceptor blocking activity (2–4). To date, GLC and high-performance liquid chromatographic (HPLC) methods involving the simultaneous detection of both 4-hydroxypropranolol and propranolol (5–12) have not been satisfactory. Sample preparation has not been uniform, and the selection of suitable fluorometric detection systems and the conditions for chromatography have varied considerably.

The present method provides a sensitive HPLC assay for the simultaneous measurement of propranolol and its active metabolite. Preliminary pharmacokinetic studies on the administration of conventional and slow-release oral propranolol formulations were conducted.

EXPERIMENTAL

Materials—4-Hydroxypropranolol[1], propranolol hydrochloride[2], labetalol hydrochloride[3], ether[4], sodium metabisulfite[5], sodium carbonate[5], and monobasic potassium phosphate[6] were used.

[1] Courtesy of Dr. C. Proctor, ICI Pty. Ltd.
[2] Sigma Chemical Co., St. Louis, MO.
[3] Gift from Glaxo Australia Pty. Ltd.
[4] May & Baker, Dagenham, England.
[5] Merck, Darnstadt, West Germany.
[6] Ajax Chemicals, Sydney, Australia.

Apparatus—A constant-flow high-performance liquid chromatograph[7] was equipped with a universal injector[8], a fluorometer[9], and a spectrofluorometer[10]. The stainless steel 30-cm long × 3.9-mm i.d. column was obtained prepacked[11].

Chromatography—The mobile phase consisted of 50% methanol[12] in 10 m*M* monobasic potassium phosphate buffer adjusted to pH 3.4 with 5 *M* HCl. It was pumped at a flow rate of 1.5 ml/min (1500 psi).

The retention volumes for 4-hydroxypropranolol, labetalol, and propranolol were 6.0, 7.2, and 9.0 ml, respectively.

The column eluate was monitored on a fluorometer set at excitation[13] and emission[14] wavelengths of 310 and 380 nm, respectively, which were optimal for 4-hydroxypropranolol fluorescence. When a spectrofluorometer was used, the wavelengths were set to be optimal for propranolol, at 295 and 360 nm, respectively. The bandpass was 20 nm. When propranolol levels were >10 ng/ml, a second fluorometer using excitation and emission wavelengths of 310[13] and 334[15] nm, respectively, occasionally was substituted for the spectrofluorometer.

Sensitivity settings on the fluorometer usually were maintained on high gain and lamp power settings; attenuations varied from ×2 to ×5 while the spectrofluorometer sensitivity range (0.3–1.0 μA) gave a full-scale deflection.

Standards—Fresh solutions of propranolol and the internal standard, labetalol, were prepared weekly as aqueous solutions at concentrations of 1 and 4 μg/ml, respectively. Solutions of 4-hydroxypropranolol were prepared daily in water containing 1 m*M* HCl and 2% (w/v) sodium metabisulfite. For preparation of plasma standards, drug-free plasma was spiked with known amounts of aqueous stock solutions of both 4-hydroxypropranolol and propranolol in the 0–2000-ng/ml of plasma concentration range.

Controls—Control heparinized plasma containing 60 ng of 4-hydroxypropranolol/ml and 100 ng of propranolol/ml was prepared and stored until use in 1-ml aliquots at −20°.

Procedure—Labetalol (400 ng, 100 μl) was added to chromium trioxide-washed glass tubes containing 1 ml of unknown plasma, plasma standards, or control plasma. To each tube, 100 μl of freshly prepared sodium metabisulfite (20% w/v), 1 ml of sodium carbonate (1 *M*, pH 10.2), and 8 ml of ether then were added in turn. The tubes were stoppered, gently shaken for 10 min on a reciprocating automatic shaker[16], and centrifuged at 2000 rpm for 10 min. The ether phase was transferred to a fresh tube and evaporated to dryness under a gentle nitrogen stream in a water bath kept at ambient temperature. The residue was reconstituted with 100 μl of chromatography mobile phase, transferred to a microfuge tube[17], and centrifuged[18] for 4 min. An aliquot of the supernate (50 μl) then was injected into the liquid chromatograph.

Quantitation—Calibration curves were constructed for both 4-hydroxypropranolol and propranolol by calculating the peak height ratios of both components to the peak height of labetalol and plotting these ratios against the amount of standard added to each tube. For routine

[7] M6000A, Waters Associates, Sydney, Australia.
[8] U6K, Waters Associates.
[9] Fluorichrom, Varian Pty. Ltd.
[10] Farrand Optical Co., Valhalla, N.Y.
[11] μBondapak C_{18}, Waters Associates.
[12] Liquid chromatography grade, Waters Associates.
[13] Filter 310 I, Varian Pty. Ltd.
[14] Filters 3.75 and 4.76, Varian Pty. Ltd.
[15] Filter 334 I, Varian Pty. Ltd.
[16] Paton Industries, South Australia, Australia.
[17] Eppendorf, Hamburg, West Germany.
[18] Mettich, Tuttlingen, West Germany.

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Table I—Assay Parameters for Measurement of 4-Hydroxypropranolol and Propranolol from Plasma

Parameter	4-Hydroxy-propranolol	Propranolol
Detection limit, ng	2	2
Recovery, %	75 ± 4 (n = 20)	68 ± 3 (n = 23)
Reproducibility within-day, %	6 (n = 5)	7 (n = 5)
Reproducibility day-to-day, %	21 (n = 27)	12 (n = 19)

assays, the peak height ratios of drug to the internal standard obtained from a plasma standard containing 50 ng of 4-hydroxypropranolol/ml, 100 ng of propranolol/ml, and 400 ng of labetalol/ml were used to calculate the concentrations of propranolol and 4-hydroxypropranolol in unknown plasma. An aliquot of control plasma was included in each batch to ensure day-to-day reproducibility.

Clinical Study—Six healthy volunteers, mean age of 21.8 ± 0.2 years and weight of 62 ± 4 kg, participated in the double-blind crossover study. After an overnight fast, each subject was given a single oral dose of propranolol. Three subjects were given a conventional 160-mg formulation, and the other three were given a slow-release 160-mg formulation. Venous blood samples (10 ml) were taken prior to drug administration and then at 0.33, 0.66, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 12, and 24 hr. One week later, the same volunteers were given the other formulation of propranolol (160 mg of conventional or slow-release formulation) and a similar set of blood samples were taken.

Pharmacokinetic Analysis—Area under the concentration–time curves were calculated by the trapezoidal method and extrapolated to infinity by dividing the final concentration point (24 hr) by the slope of the terminal phase.

RESULTS AND DISCUSSION

Both propranolol and 4-hydroxypropranolol exhibit natural fluorescence, allowing their separation by liquid chromatography and detection with a fluorescence detector. The excitation wavelengths (205 or 295 nm) are the same for both compounds, but the fluorescence emission spectra of propranolol and 4-hydroxypropranolol differ in that the wavelength maximum for emission in aqueous solution are 360 and 435 nm, respectively. Thus, there is a need for compromise in setting the wavelength of the detector to balance the loss of sensitivity for propranolol when the wavelength chosen is maximal for its metabolite.

Table II—Stability of 4-Hydroxypropranolol and Propranolol in Serum when Stored Frozen at −20°

Weeks of Storage	4-Hydroxypropranolol, ng/ml	Propranolol, ng/ml
1	66	88
2	59	79
3	61	94
4	60	91
5	64	90
9	62	90
Mean (SD)	62 ± 3 (n = 21)	90 ± 3 (n = 19)

Previous assay methods partially overcame this problem by using deuterium light sources, setting the excitation wavelength at 205 nm (5), and introducing a 340-nm cutoff filter for emission. Other assays (7, 9) using inefficient fluorometers suffered from inadequate sensitivity. Some investigators (8, 11) overcame the sensitivity problem by measuring each compound separately. This time-consuming process required injecting each extract twice, once at the chosen wavelengths for 4-hydroxypropranolol and then with the detector set at wavelengths optimal for propranolol. A recent study (12) avoided multiple injections; but to detect the 4-hydroxypropranolol and propranolol peaks, it was necessary to change the emission wavelength manually during each chromatographic run. This procedure is not suited for processing more than a few samples and does not lend itself to automatic injection techniques.

The present assay allows simultaneous detection of both compounds using an inexpensive tungsten light source. A filter fluorometer with an interference filter for the excitation light (310 nm) was used in conjunction with a combination of cutoff filters for emission, thus allowing light with a wavelength of >380 nm to be transmitted. This filter combination was optimal for 4-hydroxypropranolol fluorescence but still allowed satisfactory measurement of propranolol. The detection limit for 4-hydroxypropranolol was 2 ng/ml of plasma; for propranolol, it was 20 ng/ml (Table I). At these concentrations, peaks three times the baseline noise were obtained.

When more sensitive conditions were required for propranolol, a spectrofluorometer was connected in series with the fluorometer and set at wavelengths optimal for propranolol (295 nm/360 nm). Under these

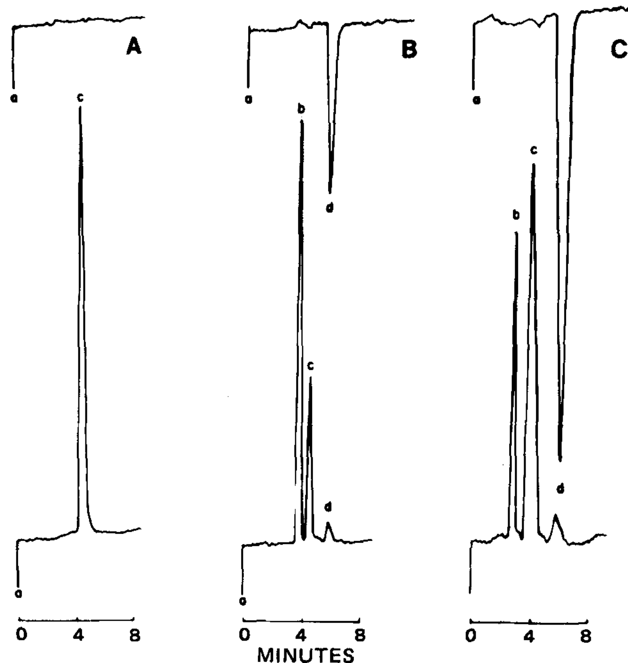

Figure 1—*High performance liquid chromatographs for: (A) a blank plasma extract, (B) an aqueous injection of 50-ng amounts of 4-hydroxypropranolol, propranolol, and (C) an extract of a patient plasma sample after receiving a single oral dose of conventional propranolol (propranolol level = 229 ng/ml; 4-hydroxypropranolol level = 47 ng/ml). Key: a, injection site; b, 4-hydroxypropranolol; c, labetalol; and d, propranolol.*

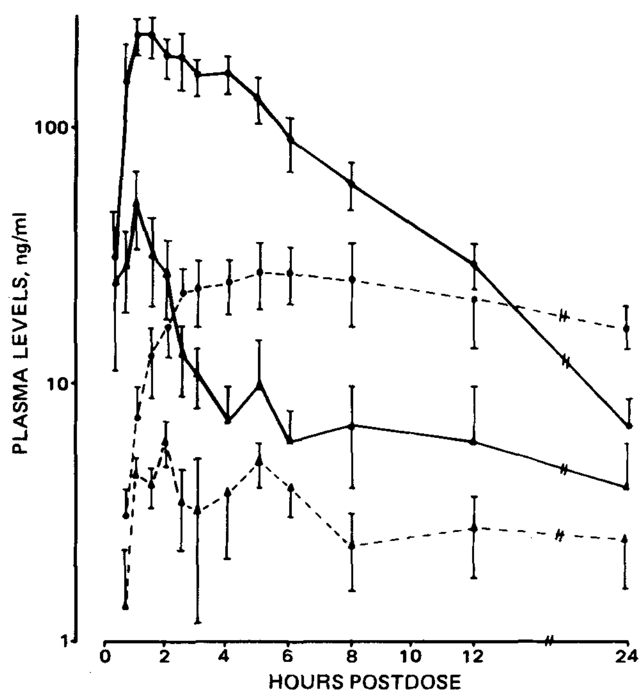

Figure 2—*Semilogarithmic plot of the mean plasma 4-hydroxypropranolol and propranolol levels versus time following single oral doses of a conventional propranolol formulation (160 mg) and a slow-release preparation (160 mg). Key: ●—●, propranolol (conventional); ●---●, propranolol (slow release); ▲—▲, 4-hydroxypropranolol (conventional); and ▲---▲, 4-hydroxypropranolol (slow release).*

conditions, the detection limit for propranolol was 2 ng/ml of plasma. 4-Hydroxypropranolol was not detected in the latter system due to the relatively narrow bandpass (20 nm) on the spectrofluorometer and the higher emission wavelengths for this compound.

If a spectrofluorometer is not available, a second fluorometer can be set at 310 nm/334 nm with interference filters; a sensitivity of 10 ng/ml for propranolol is obtained.

Methanol–10 m*M* phosphate buffer, pH 3.4 (50:50), was used as the mobile phase for reversed-phase chromatography on this bonded column and gave excellent resolution between 4-hydroxypropranolol, labetalol, and propranolol with retention volumes of 6.0, 7.2, and 9.0 ml, respectively. This separation was more reproducible than that reported with other published mobile phases, *e.g.*, acetic acid (7–10, 13) or phosphoric acid (6, 12, 14), and less harmful to the column than heptane–sulfonic acid (8).

Conjugation as the glucuronidate or sulfate is a major route of elimination of both propranolol and 4-hydroxypropranolol (15). Although the present assay method was designed primarily to measure the biologically active unconjugated drug, modification of the procedure utilizing an enzyme-catalyzed hydrolysis step prior to extraction with ether allows measurement of total propranolol and 4-hydroxypropranolol. This procedure has been well characterized (9, 12).

Figures 1A and 1B show liquid chromatograms for a blank plasma extract and an aqueous injection of 50-ng amounts of each compound. Figure 1C represents a patient plasma extract with 4-hydroxypropranolol and propranolol levels of 47 and 229 ng/ml, respectively. The upper tracing in each case represents the tracing for propranolol obtained on the spectrofluorometer. No other peaks interfered with the peaks for propranolol and 4-hydroxypropranolol. However, prazosin and quinidine interfered with the peaks for the internal standard. In the presence of either drug, 4-methylpropranolol is a suitable alternative internal standard (6).

Calibration curves were constructed by spiking drug-free plasma with aqueous solutions in the concentration range of 0–100 ng/ml for 4-hydroxypropranolol and of 0–2000 ng/ml for propranolol. The peak heights of both drugs were divided by the peak height for labetalol (internal standard) and plotted against concentration. Both drugs exhibited linear calibration curves with intercepts through the origin.

Reproducibility for both drugs was evaluated by performing replicate assays of the control plasma containing both drugs. Within-day variability was 6 and 7% for 4-hydroxypropranolol and propranolol, respectively. When the same control plasma was assayed over 5 weeks, values of 21 and 12% were obtained (Table I). The stability of 4-hydroxypropranolol was cited as a major problem (5, 8), so although sodium metabisulfite was added as a stabilizer to the plasma prior to extracting, it was feared that the plasma stored frozen without stabilizer might deteriorate with time. When control plasma was assayed at weekly intervals over 9 weeks, no deterioration was evident (Table II). This result was confirmed by Taburet *et al.* (8), who found that the metabolite was stable for at least 4 weeks at $-20°$.

No data have been published on plasma level profiles of both unconjugated propranolol and 4-hydroxypropranolol following oral administration of a slow-release propranolol formulation. The mean ($\pm SEM$) plasma concentrations of these two compounds were compared in six volunteers receiving, on separate occasions 1 week apart, a single oral 160-mg dose of propranolol in a conventional formulation and a 160-mg slow-release preparation (Fig. 2). Peak levels of propranolol after the conventional formulation were 236 ± 38 ng/ml at 60 min, consistent with previous reports (7, 16). Peak levels for the slow-release preparations were considerably lower and delayed (28 ± 8 ng/ml, obtained 5 hr postdose).

The slow-release formulation was not associated with an increased rate of formation of the active metabolite 4-hydroxypropranolol. In fact, in all patients, peak levels (conventional, 51 ± 17 ng/ml; slow release, 6 ± 1 ng/ml) and area under the curve values (*AUC*) (conventional, 218 ± 17 ng hr/ml; slow release, 141 ± 32 ng hr/ml) for the 4-hydroxy metabolite were considerably less following single doses of the slow-release formulation. The persistence of propranolol in plasma for >24 hr with the slow-release formulation suggests that drug accumulation may occur with chronic daily therapy and that attainment of steady state may require several days of treatment. Calculation of the bioavailability for the slow-release preparation by extrapolation of the 24-hr data is likely to overestimate the *AUC*. Nevertheless, bioavailability of the slow-release preparation was poor; corresponding propranolol *AUC* estimations suggest that the bioavailability of single doses of the slow-release formulation was at least 2.9 times lower than that of the conventional preparation. The association of low propranolol levels and low levels of the 4-hydroxy metabolites is consistent with the suggestion that high 4-hydroxypropranolol levels are formed only in the presence of high concentrations of the parent drug in the liver (17).

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