Improved Liquid-Chromatographic Determination of Propranolol in Plasma, with Fluorescence Detection

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We describe an analysis for propranolol in plasma, with use of reversed-phase "high-pressure" liquid chromatography and fluorescence detection. Pronethanol is used as the internal standard. The procedure, which involves extraction into an organic solvent, evaporation, and clean-up by micro-scale back extraction from hexane into an aqueous phase, is specific and sensitive. The detection limit is <6 μg/L (2.3 × 10⁻⁸ mol/L). Within-day and between-day coefficients of variation are 1.8 and 4.4%, respectively. Commonly used drugs, including procainamide, N-acetylprocainamide, and quinidine, do not interfere.

Additional Keyphrases: "high-pressure" liquid chromatography • drug assay • monitoring therapy • heart disease

Propranolol ("Inderal," Ayerst), a beta-adrenergic blocker, is widely used to treat angina pectoris, certain cardiac arrhythmias, and hypertension. Although the clinical significance of plasma drug concentrations in various disease states is not yet completely defined, therapeutic monitoring of propranolol is of value in selected patients, and requests for this assay are likely to increase.

The procedure most widely used for measuring propranolol in plasma has been the double-extraction fluorometric technique of Shand et al. (1). Although this procedure has been criticized for the lack of specificity inherent to this type of assay (2), particularly at low concentrations, it has been used in most published clinical-research studies. Considerably better sensitivity and specificity has been achieved by using gas chromatography with electron capture detection (3-5), procedures that include fairly involved derivitization steps. Finally, as with most other drug assays, mass fragmentography (6) is the most advanced technique, but is neither economically nor technically feasible for routine use in most clinical laboratories.

In the method we describe here, we used reversed-phase "high-pressure" liquid chromatography (HPLC) with fluorescence detection. This procedure is as sensitive as, and considerably less complex than, gas chromatography-electron capture procedures, and it is more sensitive and specific than fluorescence procedures that do not involve chromatographic separation. We believe, for reasons discussed herein, that it also offers advantages over other recently reported HPLC procedures (7-9).

Materials and Methods

Apparatus

A Model 601 liquid chromatograph (Perkin-Elmer Corp., Norwalk, CT 06856) containing a 4.6 mm × 25 mm Partisil 10 ODS reversed-phase column (Whatman, Inc., Clifton, NJ 07014) was used with a 204-S fluorescence detector (Perkin-Elmer) and Model 355 (Linear Instruments Corp., Irvine, CA 92714) strip-chart recorder. A Perkin-Elmer Model LC-55 variable-wavelength detector connected in tandem with the fluorometer was used for some studies.

Reagents and Standards

All glassware was acid washed with 1.0 mol/L HCl. All chemicals were of reagent grade unless otherwise indicated. Propranolol and pronethanol, as the hydrochloride salts, were gifts of Ayerst Laboratories, New York, NY 10016.

Stock standards (2.0 g/L, calculated as the base) of propranolol and pronethanol were prepared in methanol in about 5-mL quantities and stored in a freezer (−15 to −20 °C). These are stable for at least one month.

Working propranolol standards in plasma were prepared at the time of analysis. A fresh 100-fold aqueous dilution of the stock standard was used to supplement drug-free plasma to the desired propranolol concentrations.

A working solution of internal standard (40 mg/L) was prepared at the time of analysis by diluting the stock pronethanol standard 50-fold in water.

Carbonate buffer (1.0 mol/L, pH 9.6) was prepared from Na₂CO₃ and NaHCO₃.

Phosphate buffer (0.1 mol/L, pH 2.7) was prepared from KH₂PO₄ and H₃PO₄.

Acetonitrile and hexane, glass-distilled and ultraviolet grade, were obtained from Burdick Jackson Lab., Muskegon, MI 49442. Reagent-grade isoamyl alcohol was redistilled in our laboratory.

The extraction solvent was hexane/isoamyl alcohol, 98.5/1.5 by vol.

Procedure

Blood was drawn in heparinized glass syringes, and the plasma frozen until assayed. We mixed 2.0 mL of plasma (sample or standard), 40 μL of working internal standard so-
lution, and 1.0 mL of carbonate buffer in 15-mL screw-capped (Teflon-lined caps) culture tubes. After extraction with 10 mL of hexane/isoamyl alcohol, and centrifugation, about 8 mL of the solvent (upper) phase was transferred with Pasteur pipettes to clean test tubes, dehydrated over anhydrous Na2SO4, decanted into 12-mL conical centrifuge tubes, and evaporated under nitrogen. The residue was dissolved in 50 µL of phosphate buffer, 0.5 mL of hexane was added, and the mixture was shaken and centrifuged. All of the aqueous (lower) phase was transferred to a “nipple” tube (Concentratubes; Laboratory Research Co., P.O. Box 36509, Los Angeles, CA 90836) and after a brief centrifugation, 20 µL of the aqueous phase was chromatographed.

Chromatographic conditions: For elution we used acetonitrile/phosphate buffer (pH 2.7), 25/75 by volume, at a flow rate of 1.5 mL/min and oven temperature of 50 °C. The activation and emission monochromators of the fluorometer were set to 285 and 350 nm, respectively. Additionally, a Corning no. 7-54 ultraviolet filter was used in the activation beam, and a Corning no. 0-54 320-nm cut-off filter was placed in the emission beam. When used, the ultraviolet detector was set to 210 nm.

Results

Pronethalol (the internal standard) and propranolol had retention times of 4.8 and 6.3 min, respectively. Figure 1 shows chromatograms obtained from a drug-free plasma, a standard in plasma, and plasma from a patient receiving propranolol.

Relative peak height was linearly related to plasma propranolol concentrations over the range 6 to 400 µg/L (2.3 × 10⁻⁸ to 1.5 × 10⁻⁶ mol/L). Statistical analysis of one such curve showed a correlation coefficient of 0.99 (n = 7) and the least-squares fit (x = concentration, µg/L; y = peak height ratio) was expressed by the equation y = 0.0065x + 0.016.

Within-day reproducibility (CV) for a concentration of 130 µg/L was 1.90% (n = 10). The between-day reproducibility (CV) for 12 frozen aliquots, thawed and analyzed during a three-week period, was 4.5%.

Uncorrected recovery of propranolol and pronethalol, as assessed by comparing peak heights of extracted standards with that of unextracted compound, was 86 and 75%, respectively. This incomplete recovery was compensated for by using extracted standards in plasma for calibration. Although pronethalol recovery was comparable to that of propranolol, higher concentrations were used to compensate for its weaker fluorescence.

Propranolol-free plasma (n = 13) showed no peaks that might interfere with measurement of propranolol or pronethalol. Minor baseline deflections were generally equivalent to less than 1 µg of propranolol per liter. Icteric, hemolyzed, or lipemic specimens showed no interfering peaks. The following drugs (200-ng injections) were evaluated and did not interfere: procainamide, N-acetylprocainamide, caffeine, nicotine, theophylline, thioridazine, thalozene, diazepam, nordiazepam, oxazepam, chlordiazepoxide, nordiazepam, demoxepam, amitriptyline, nortriptyline, imipramine, desipramine, meperidine, and diphenhydramine. Only procainamide and N-acetylprocainamide produced detectable peaks of fluorescence.

Although the monochromator settings were far from optimal for quinidine, at the much higher concentrations which this drug may attain in plasma, its peak did overlap with that of pronethalol (0.4-min difference in retention time). A decrease in the proportion of acetonitrile concentration in the mobile phase to 20% by volume increased the retention-time difference to 1 min. With these modified conditions, even “toxic” concentrations of quinidine did not interfere. Interfering quinidine metabolites were not seen when we tested samples of plasma from patients receiving this drug.

Discussion

The clinical significance of plasma propranolol concentrations and indications for therapeutic monitoring are not as clear as with several other cardiac drugs. Although complete peripheral β-adrenergic blockade is reported (10, 11) when concentrations of about 100 µg/mL (3.9 × 10⁻⁷ mol/L) are reached, higher concentrations are reported in therapy for some forms of hypertension (12, 13).

Propranolol does have a metabolite, 4-hydroxypropranolol, which is produced in the liver and has been shown to have activity as a β-adrenergic blocker in animals and, indirectly, in humans (14, 15). This metabolite is not evident in the plasma after intravenous administration of propranolol, but its apparent effect is detectable shortly after oral therapy. Walle et al. (6) and others (14) have noted that 4-hydroxypropranolol is extremely unstable, and the former workers recommend addition of sodium bisulfite to plasma to prevent deterioration of this metabolite. Although, in our hands, the unextracted compound showed good liquid-chromatographic behavior, we found that analytical recovery from plasma was too variable and (or) losses too unpredictable, even with use of more polar extraction solvent and even after addition of sodium bisulfite, for us to recommend its measurement at this time. A report of the HPLC measurement of 4-hydroxypropranolol by Nation et al. (9) provided no data on samples from patients to support it, and showed only a chromatogram after intravenous dosage when, as expected, no metabolite could be detected. In almost all published clinical studies, including the most recent, only the parent compound has been
metabolite concentrations measured. It has been suggested that measurement of the metabolite may not be clinically important because of its low concentrations and much shorter biological half-life as compared to that of the parent drug (10, 11). Pine et al. (10) and Cleveland and Shand (11) have reported that after chronic oral therapy all of the β-blocking activity can be accounted for by the propranolol concentration.

We only evaluated sensitivity to a low concentration, of 6 µg/L (2.3 × 10^-8 mol/L), which probably is well below concentrations of therapeutic interest. A satisfactory peak was obtained at this concentration, with use of fluorometer settings more than 10-fold less sensitive than the maximum achievable, suggesting that lower concentrations can be detected if desired. We initially evaluated spectrophotometric detection for the chromatographic analysis of propranolol. However, fluorometry was considerably more sensitive and yielded cleaner chromatograms.

Recently, Wood et al. (7) reported a fluorescence HPLC procedure for the analysis of propranolol. Their procedure, in which use of 4 mL of plasma is specified, does not involve any further clean-up after solvent evaporation. Our brief clean-up step, involving a micro-scale back extraction into acid buffer before chromatography, added little to the time required for or complexity of the assay and resulted in cleaner chromatograms. We believe that a clean-up step to remove neutral compounds is highly desirable when submicrogram concentrations of drugs are to be measured. The procedure published by Mason et al. (8), similarly does not involve a clean-up step, nor does it make use of an internal standard. Moreover, the separation is achieved with a polar-phase bonded column, which requires elution with large volumes of organic solvent as compared to reversed-phase techniques. Immediate back extraction from ethyl acetate into an aqueous phase, as suggested by Nation et al. (9), resulted in much poorer recovery of propranolol in our hands. Mason et al. (8) and Nation et al. (9) utilized a lower, less-selective wavelength for activation, conditions under which interferences are more likely. For all these reasons we believe that our procedure is better optimized for trouble-free therapeutic monitoring.

We avoid the use of commercial evacuated tubes for specimen collection because of the reported interference from tris(2-butoxyethyl) phosphate, which is eluted from some rubber stoppers (16, 17). This plasticizer reportedly (17) displaces propranolol (and various other basic drugs) from binding proteins, with subsequent uptake by erythrocytes, resulting in spuriously low values for concentrations in plasma. Recently available tubes with stoppers claimed to be free of this compound may be suitable for specimen collection.

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References