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DETERMINATION OF PROPRANOLOL AND ITS MAJOR METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITHOUT SOLVENT EXTRACTION

MANWAI LO and SIDNEY RIEGELMAN*

School of Pharmacy, University of California, San Francisco, CA 94143 (U.S.A.)

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SUMMARY

Fast, reliable, specific and sensitive methods are reported to accurately quantitate unchanged propranolol in plasma, and its major metabolites in plasma and urine after enzymatic hydrolysis without the need for solvent extraction. These methods enable the analyst to process a large number of propranolol samples in one working day and should prove valuable to clinical laboratories demanding both speed and specificity in an assay.

INTRODUCTION

Propranolol, a drug widely used in the treatment of hypertension, angina, cardiac arrhythmias and thyrotoxicosis is almost completely metabolized in man [1]. The major metabolites identified in plasma and urine include: conjugates of propranolol and 4-hydroxypropranolol and free α-naphthoxylactic acid [2—7]. Methods presently available to measure these metabolites use either spectrofluorometry [8], thin-layer chromatography [2,9], gas chromatography [10—13], gas chromatography—mass spectrometry [3,14,15] or high-performance liquid chromatography (HPLC) [4,16—20]. All these methods require extensive sample work-up. We report for the first time an assay procedure that requires only a simple protein precipitation step yet allows one to measure all these compounds in one chromatographic run. For clinical laboratories not concerned with measuring metabolites, a method is reported which affords an accurate measure of unconjugated propranolol in plasma.

EXPERIMENTAL

Standards and reagents

Propranolol HCl, 4-hydroxypropranolol HCl, α-naphthoxylactic acid, propranolol glycol and N-desisopropylpropranolol were kindly supplied by I.C.I. (Macclesfield, Great Britain). α-Naphthol and α-naphthoxyacetic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Trans World Chemicals (Washington, DC, U.S.A.), respectively.

The internal standard N-ethylpropranolol was prepared according to the method of Wood et al. [21]. However, the crude product contained an impurity that interfered with the assay and could not be separated by solvent extraction. Therefore it was necessary to purify the product by reversed-phase HPLC and solvent extraction. The recovered N-ethylpropranolol was stored in methanol, which was used to make up three concentrations for use as internal standards: one in acetonitrile, for unchanged propranolol in plasma and two in dilute phosphoric acid, for hydrolyzed plasma and at five-fold concentration for use in the urine assay.

Acetonitrile (UV grade) and methanol were supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were analytical grade.

Instrumentation

A Varian Model 8500 high-performance liquid chromatograph equipped with a Perkin-Elmer 650-10 LC fluorescence spectrophotometer, a LiChrosorb RP-8 column (25 cm × 4.6 mm I.D.; 10 μm particle size; Altex Assoc., Berkeley, CA, U.S.A.) and a LiChrosorb RP-2 precolumn (4 cm × 3.2 mm I.D.; 10 μm particle size; Altex) were used. The fluorescence output was recorded on a dual-channel recorder (Linear Instruments, Irvine, CA, U.S.A.). Injections were made with a 100-μl Hamilton syringe through a Valco CV-6-UHPa-N60 sweep-flow injector equipped with a 100-μl loop.

Method 1: Measurement of unconjugated propranolol in plasma

Daily standard curves were prepared as follows. A 1.5 μg/ml propranolol (free base) solution in water was prepared from a 50 μg/ml propranolol HCl aqueous stock solution. A 0.5-ml aliquot of this standard solution was added to 2 ml of drug-free human plasma to make up a 300 ng/ml standard. It was then serially diluted with plasma to yield concentrations of 150, 75, 30, 20, 10 and 5 ng/ml.

Plasma samples were processed by transferring a 0.2-ml quantity into an Eppendorf polypropylene 1.5-ml micro test tube (Brinkmann No. 2236411-1, Brinkmann Instruments, Westbury, NY, U.S.A.), and 0.4 ml of the N-ethylpropranolol solution in acetonitrile was added. After the sample was vortexed for 15 sec it was centrifuged for 2 min at 12,800 *g* using an Eppendorf Microcentrifuge, Model 5412. The clear supernatant was transferred to a disposable glass culture tube (13 × 100 mm) and evaporated to an approximate volume of 0.1—0.2 ml under a gentle stream of nitrogen. After adding 0.2 ml of 0.05 *M* phosphoric acid and brief vortexing, a 50—90-μl aliquot was injected onto the column. The mobile phase was composed of 360 ml acetonitrile, 180 ml methanol and 70 ml of 0.0871 *M* phosphoric acid diluted to one liter with glass

distilled water. The flow-rate was 100 ml/h. The fluorometer was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm. Both slit openings were set at 20 nm. The fluorometer was operated at a sensitivity range of 0.1 and normal power gain. The output was 1 V and sensitivity varied by changing the voltage spans on the dual-pen recorder. One pen was always fixed to measure the internal standard.

Method 2: Measurement of propranolol, 4-hydroxypropranolol and α-naphthoxylactic acid in plasma and urine after enzymatic hydrolysis

The plasma and urine standards were made up as follows: the most concentrated plasma standard containing 2 μg/ml of α-naphthoxylactic acid, 1 μg/ml of propranolol and 4-hydroxypropranolol was prepared by evaporating to dryness 0.5 ml of 20 μg/ml α-naphthoxylactic acid in methanol. Then 0.5 ml of propranolol (10 μg/ml in water) and 0.25 ml of 4-hydroxypropranolol (20 μg/ml in 0.01 *M* phosphoric acid including 5 mg/ml ascorbic acid to minimize oxidation) were added. These were diluted with 4.25 ml of human (drug-free) plasma. Additional standards were prepared by serial dilution with more blank plasma. Urinary standards were prepared slightly differently because of the instability of 4-hydroxypropranolol in urine. A urine sample containing 20 μg/ml of propranolol and α-naphthoxylactic acid was first prepared and then serially diluted with more blank urine. A series of disposable culture tubes were prepared each containing 0.1 ml of a 200 mg/ml solution of ascorbic acid. Then 0.025, 0.05, 0.1, or 0.2 ml of 4-hydroxypropranolol (20 μg/ml protected by ascorbic acid) was added. To each of the tubes was added 0.2 ml of the appropriate propranolol—α-naphthoxylactic acid mixture and a 0.2-ml quantity of the 5-fold concentrated internal standard solution.

The assays were performed as follows: a 0.2-ml quantity of urine or a 0.4-ml quantity of plasma were mixed with 0.2 ml aqueous internal standard in a disposable glass culture tube. The urine sample was diluted with 0.2 ml of water. A 0.1-ml quantity of ascorbic acid (200 mg/ml), 0.04 ml of acetate buffer (1.4 *M*, pH 5.5) and 25 mg of β-glucuronidase/aryl sulfatase (400 units/mg, Sigma G 0751, St. Louis, MO, U.S.A.) were added. The mixture was incubated at 37°C for 90 min. After precipitating the protein with 0.8 ml of acetonitrile, the resulting mixture was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 3 min at 12,800 *g*. A 0.6-ml amount of the clear supernatant was removed and 0.3 ml of 0.05 *M* phosphoric acid was added. It should be noted that no evaporation was necessary. A 40—50-μl aliquot was injected onto the column. The eluent was composed of 300 ml of acetonitrile, 90 ml of methanol and 66 ml of 0.0871 *M* phosphoric acid diluted to one liter with glass distilled water. The flow-rate was 100 ml/h. Since 4-hydroxypropranolol fluoresces differently when compared to propranolol and α-naphthoxylactic acid, the fluorometer's emission wavelength was first set to 430 nm to measure 4-hydroxypropranolol. The excitation wavelength was fixed at 310 nm. After the elution of 4-hydroxypropranolol (about 6 min), the emission was then changed to 350 nm for the detection of propranolol and α-naphthoxylactic acid. Slit widths were set at 20 nm and the sensitivity range set at 0.1. The recorder output was set at 1 V and a normal power gain was used. The spans of the recorder were used to vary sensitivity.

By omitting the enzymatic hydrolysis procedure one can directly measure α-naphthoxylactic acid and unconjugated propranolol. For these assays the fluorescent excitation wavelength was set at 230 nm and the emission at 340 nm.

RESULTS AND DISCUSSION

Fig. 1 includes chromatograms of blank plasma and a typical patient plasma obtained by the direct injection assay. The total elution time per assay is 8 min. All the known metabolites of propranolol elute before propranolol and do not interfere with the assay. The limit of detection using 0.2 ml of plasma is 2 ng/

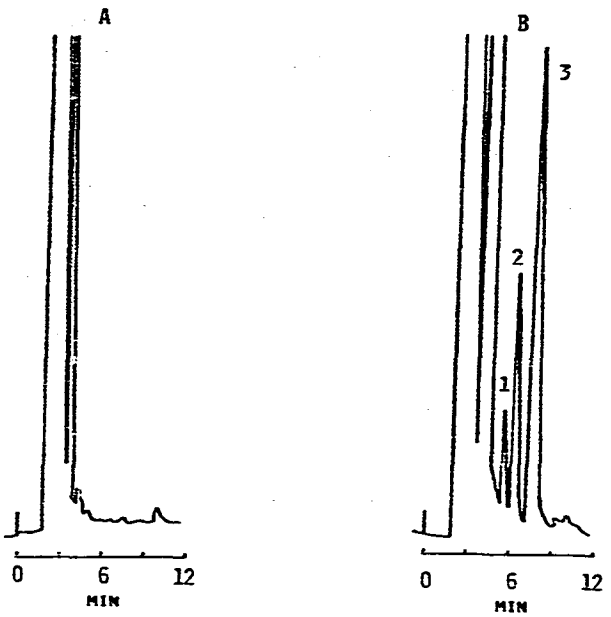

Fig. 1. Assay of unchanged propranolol in plasma (see Method 1). (A) Drug-free human plasma. (B) 2-h plasma sample obtained from an angina patient taking 20 mg of propranolol every 6 h. Peaks: 1 = α-naphthoxyacetic acid; 2 = propranolol, 34 ng/ml; 3 = N-ethyl-propranolol (internal standard). Fluorometer settings: excitation = 230 nm; emission = 340 nm.

TABLE I

INTRA- AND INTER-ASSAY VARIATION OF PROPRANOLOL IN PLASMA

Spiked concentration (ng/ml)	Intra-assay C.V. (%) (n = 5)	Inter-assay C.V. (%) (n = 3, over 2 weeks)
10.05	1.5	3.9
30.14	n.d.*	3.8
75.35	0.9	3.9
150.69	2.2	n.d.*
301.38	1.1	5.5

*Not determined.

ml. The precision and accuracy of the assay are shown in Table I. Intra- and inter-assay coefficients of variation (C.V.) were 2.2%, and 5.5%, respectively, over the concentration range of 10—300 ng/ml. Our method was compared with a double extraction technique developed in this laboratory earlier [22]. It involved alkalinizing a plasma sample containing propranolol with sodium

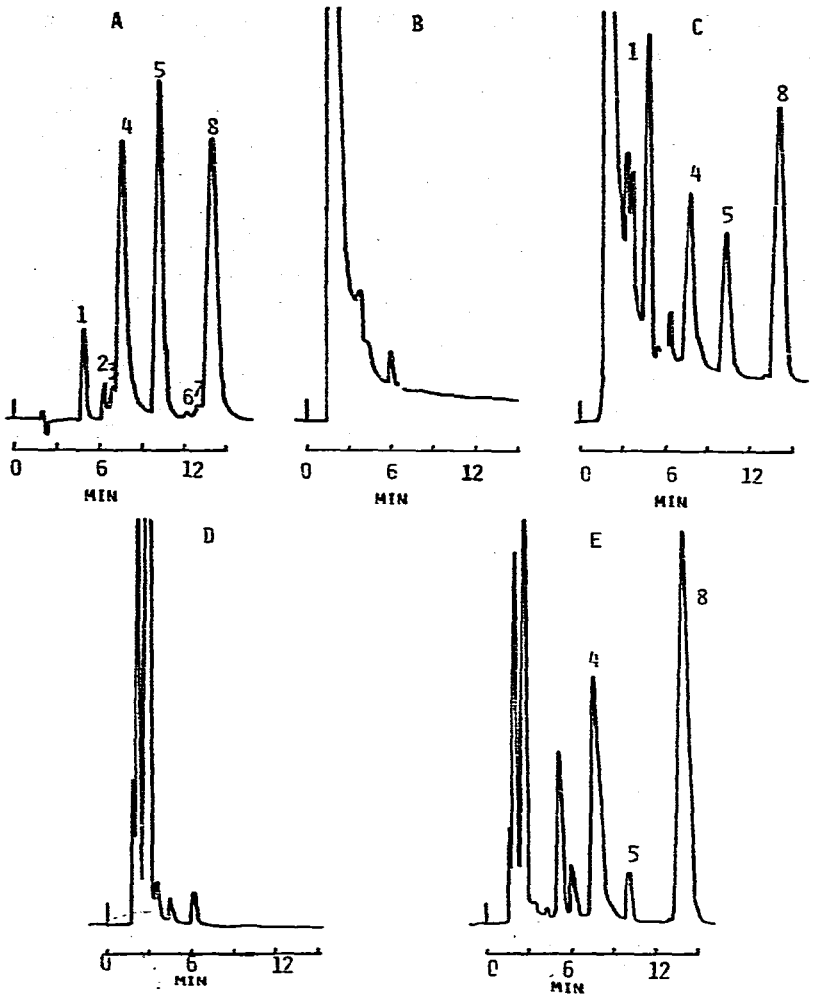

Fig. 2. Assay of propranolol and metabolites in plasma (see Method 2). (A) Standard mixture. Peaks: 1 = 4-hydroxypropranolol; 2 = N-desisopropylpropranolol; 3 = propranolol glycol; 4 = α-naphthoxylactic acid; 5 = propranolol; 6 = α-naphthol; 7 = α-naphthoxyacetic acid; 8 = N-ethylpropranolol (internal standard). (B) Hydrolyzed human plasma blank. (C) Hydrolyzed 1-h plasma sample from an angina patient taking 80 mg of propranolol every 6 h. Peaks: 1 = 490 ng/ml 4-hydroxypropranolol; 4 = 770 ng/ml α-naphthoxylactic acid; 5 = 490 ng/ml propranolol; 8 = N-ethylpropranolol (internal standard). (D) Unhydrolyzed human plasma blank (enzyme omitted). (E) Unhydrolyzed patient sample, same as C. Peaks: 4 = 770 ng/ml α-naphthoxylactic acid; 5 = 128 ng/ml propranolol; 8 = N-ethylpropranolol (internal standard). Fluorometer settings: B and C, excitation = 310 nm; emission = 430 nm for the first 6 min, then changed to 350 nm. D and E, excitation = 230 nm; emission = 340 nm.

carbonate buffer and extracting it with diethyl ether. The propranolol was then extracted into phosphoric acid and injected onto the column. Comparison of the values obtained using the two techniques to assay three samples from angina patients differed from one another by 6.4%, −3.7% and 1%, respectively, even though the analyses were done over a six-month period.

Drugs tested for interference by direct injection onto the column included hydralazine, hydrochlorthiazide, triamterene, furosemide, procainamide and quinidine. Only quinidine was found to interfere.

Approximately 75% of propranolol in plasma, virtually all propranolol in urine and 4-hydroxypropranolol in plasma and urine appear as their conjugates. Enzymatic hydrolysis is therefore required to liberate the compounds. Fig. 2A shows how 4-hydroxypropranolol, α-naphthoxylactic acid, propranolol and the internal standard N-ethylpropranolol are separated from the minor metabolites of propranolol, namely N-desisopropylpropranolol, propranolol glycol, α-naphthol and α-naphthoxyacetic acid. Chromatograms of patient plasma and urine samples and blanks after enzymatic hydrolysis are shown in Fig. 2B, C and Fig. 3. It can be seen that there is no interference from normal biological constituents. The same patient plasma sample was analyzed before hydrolysis for α-naphthoxylactic acid and unconjugated propranolol and the chromatogram is shown in Fig. 2E, again there is no interference from blank plasma (Fig. 2D).

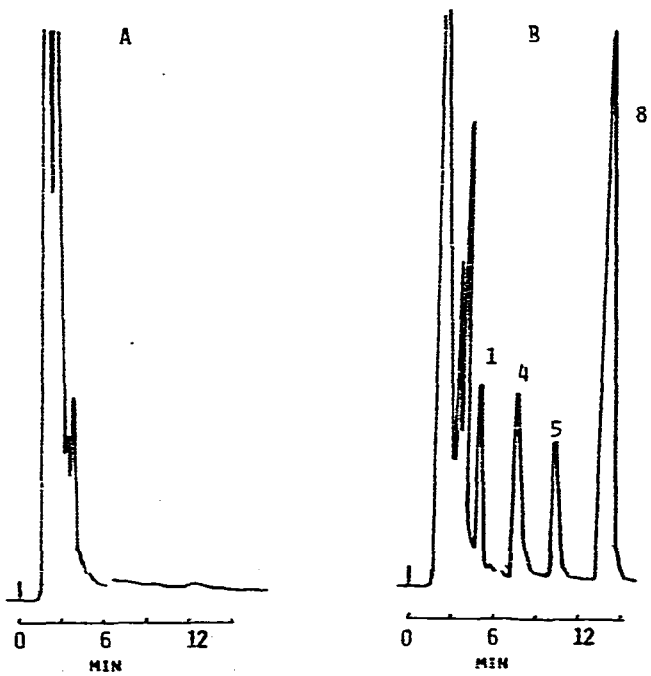

Fig. 3. Assay of propranolol and metabolites in urine (see Method 2). (A) Hydrolyzed human urine blank. (B) Hydrolyzed 2—3-h urine collection from a hypertensive patient taking 10 mg of propranolol every 6 h. Peaks: 1 = 3.3 μg/ml 4-hydroxypropranolol; 4 = 3.27 μg/ml α-naphthoxylactic acid; 5 = 2.36 μg/ml propranolol; 8 = N-ethylpropranolol (internal standard). Fluorometer settings: excitation = 310 nm; emission = 430 nm for the first 6 min, then changed to 350 nm.

Concentration of propranolol conjugates can thus be determined by the difference of the propranolol level measured before and after hydrolysis (Fig. 2C and E). The total chromatographic analysis time is 15 min. This method allows determination of all three compounds even though they have widely different acid—base characteristics. A kinetic study examining the rate of enzymatic hydrolysis revealed that 90 min was sufficient for complete hydrolysis of the conjugates using 10,000 units of enzyme. Pritchard et al. [4] used sodium metabisulfite as an antioxidant during hydrolysis. In contrast, the present method utilizes ascorbic acid. A comparison of these two antioxidants as well as sodium bisulfite was made using metabolites-spiked plasma and urine.

TABLE II

PERCENT OF PROPRANOLOL AND METABOLITES REMAINING IN PLASMA AND URINE FOLLOWING ENZYMATIC HYDROLYSIS* USING ASCORBIC ACID, SODIUM METABISULFITE, AND SODIUM BISULFITE AS ANTIOXIDANTS

Compound	Antioxidant**		
	Ascorbic acid	Sodium metabisulfite	Sodium bisulfite
Propranolol			
Plasma (0.5 μg/ml)	99.4	95.9	94.6
Urine (5.0 μg/ml)	96.7	88.3	88.6
4-Hydroxypropranolol			
Plasma (0.5 μg/ml)	99.9	76.1	72.5
Urine (5.0 μg/ml)	99.2	39.2	46.3
α-Naphthoxylactic acid			
Plasma (1.0 μg/ml)	99.6	91.3	91.5
Urine (5.0 μg/ml)	91.9	71.6	66.3

*10,000 units of enzyme, 90 min, 37°C.
**20 mg of each antioxidant used.

TABLE III

INTRA- AND INTER-ASSAY VARIATION OF PROPRANOLOL AND ITS METABOLITES IN PATIENT PLASMA AND URINE SAMPLES* FOLLOWING ENZYMATIC HYDROLYSIS**

	Propranolol	4-Hydroxypropranolol	α-Naphthoxylactic acid
Plasma			
Intra-day C.V. (%) (n=5)	1.6	2.2	2.3
Inter-day C.V. (%) (n=3)	1.3	7.1	1.9
Urine			
Intra-day C.V. (%) (n=5)	1.4	1.4	0.9
Inter-day C.V. (%) (n=3)	1.3	3.9	4.2

*Determinations were made over a 2-week, and 2-month period for plasma and urine samples, respectively.
**10,000 units, 37°C, 90 min.

Duplicate plasma standards containing 1 μg/ml of α-naphthoxylactic acid, 0.5 μg/ml of propranolol and 4-hydroxypropranolol, and urine standards containing 5 μg/ml of all three compounds were incubated with enzyme for 90 min using 20 mg of each of the three antioxidants. The percent of each compound remaining was calculated by comparison to an unincubated standard. The results shown in Table II suggest that ascorbic acid is the best antioxidant for the protection of all three compounds.

Intra- and inter-day assay variations of propranolol and its metabolites determined by assaying patient plasma and urine samples over a period of two weeks for plasma and two months for urine are shown in Table III. The limits of detection of the three compounds are about 20 ng/ml using 0.4 ml plasma and 100 ng/ml using 0.2 ml urine. Lower levels can be measured by use of larger volumes of samples.

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