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DETERMINATION OF PROPRANOLOL AND SIX METABOLITES IN HUMAN URINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of propranolol and six of its metabolites, as well as their glucuronide and/or aryl sulfate conjugates in human urine is described. Propranolol and its basic and neutral metabolites are extracted into ether at pH 9.8, evaporated to dryness, reconstituted, separated on a reversed-phase, high-pressure liquid chromatographic system and quantitated using fluorescence detection. The aqueous urine aliquot is then made acidic and the acid metabolites extracted and measured using similar methods. The presence of 2% sodium metabisulfite in all urines collected is essential to ensure the stability of 4-hydroxypropranolol during collection and storage. Preliminary data is presented from 24-h urine samples collected from three patients chronically receiving propranolol.

INTRODUCTION

The widely used β-adrenergic blocking drug, propranolol, has been shown to be more than 95% metabolized by the liver in rat, dog and man [1,2]. Many of the metabolites of propranolol have been identified in human urine by sensitive gas chromatographic—mass spectrometric techniques [3] (Fig. 1). Initial reports suggested that 4-hydroxypropranolol and naphthoxylactic acid were two major propranolol metabolites excreted by man [4]. 4-Hydroxypropranolol was subsequently shown to have β-adrenergic blocking properties equivalent to those of propranolol [5]. This metabolite has been implicated in contributing to the β-adrenergic blockade observed following oral doses of propranolol [6—8] but the quantitative formation and excretion of this metabolite relative to propranolol dose has not been clearly defined. Many of the other metabolites have pharmacological properties of their own which may contribute to some of the actions of propranolol [3].

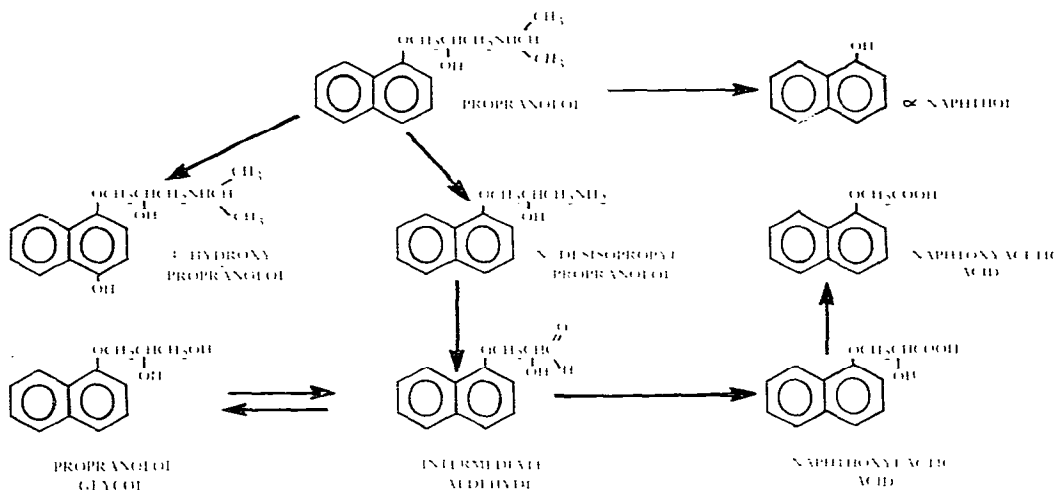*To whom correspondence should be addressed.

Fig. 1. The proposed metabolism of propranolol to the compounds measured by the HPLC assay procedure. Further metabolism of all the metabolites except α-naphthol has been postulated [3].

Studies in man using radiolabelled propranolol established that greater than 85—95% of the administered radioactivity appeared in the urine of the subjects studied [2]. Thus, the metabolites of propranolol are largely excreted via renal elimination. Therefore the urinary excretion profile of propranolol metabolites should be a reflection of the hepatic metabolism of this drug. Measurement of urinary propranolol metabolites would allow studies to be conducted concerning the effect of dose, routes of administration and disease states on the hepatic metabolism of propranolol. In view of the above considerations, a sensitive assay for determining the urinary concentrations of propranolol and six of its metabolites (Fig. 1) was developed.

EXPERIMENTAL

Standards and reagents

Propranolol HCl was obtained from Ayerst Laboratories, New York, N.Y., U.S.A. Naphthoxylactic acid and 4-hydroxypropranolol HCl were kindly supplied by I.C.I., Macclesfield, Great Britain. α-Naphthol and 2-naphthoxy-acetic acid were obtained from Aldrich, Milwaukee, Wisc., U.S.A. α-Naphthoxy-acetic acid and procainamide hydrochloride were both supplied by K&K Labs., Plainview, N.Y., U.S.A.

Propranolol glycol and N-desisopropylpropranolol were synthesized by previously described methods [3]. The melting points for propranolol glycol (95.5—96.5°, uncorrected) and N-desisopropylpropranolol (152.5—154.5°, uncorrected) were identical to those previously reported [3].

Reagent grade absolute ether was obtained from Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A. All other reagents, including high-pressure liquid chromatography (HPLC) grade methanol and acetonitrile, were supplied by Fisher Scientific, Pittsburgh, Pa., U.S.A. Aqueous reagents were prepared using

high purity water obtained from a water purification system (Hydro Service and Supply, Durham, N.C., U.S.A.).

An aqueous internal standard solution was prepared containing 50 μg/ml procainamide hydrochloride (internal standard for the assay of propranolol and its basic metabolites) and 50 μg/ml 2-naphthoxyacetic acid (internal standard for the quantitation of the acidic metabolites of propranolol).

All centrifuge tubes and volumetric flasks were silanized and washed as previously described [9].

Instrumentation

Reversed-phase HPLC was performed utilizing a Model M-6000A solvent delivery system, a U6K Universal Injector, and a C_{18} μBondapak column (30 × 4mm I.D.; 10μm average particle size; Waters Assoc., Milford, Mass., U.S.A.). The fluorescent intensity of the column eluent was measured continuously utilizing an Aminco Fluoromonitor equipped with a germicidal lamp source and a primary UV interference filter (295 nm; American Instrument Co., Silver Springs, Md., U.S.A.). The absorbance of the eluent was determined using a variable wavelength UV absorption detector set at 295 nm (Model SF770; Schoeffel, Westwood, N.J., U.S.A.). Both relative fluorescence and absorbance were recorded on a dual channel OmniScribe recorder (Houston Instruments, Austin, Texas, U.S.A.) with an input range of 1—10 mV.

Assay procedure for the measurement of unconjugated propranolol and its metabolites in human urine

Both the acidic and basic metabolites of propranolol were extracted from the same urine aliquot. Urine (1.0 ml) was added to a 15-ml screw-capped centrifuge tube containing 100 μl of 20% sodium metabisulfite, 100 μl of internal standard solution and 1.0 ml of 1 M carbonate buffer, pH 10.2. The buffered urine was extracted with 8 ml of anhydrous ether for 10 min using an Eberbach shaker at high speed. The organic and aqueous layers were then separated by centrifugation at 300 g for 5 min at 4°. A 2-ml aliquot of the ether layer was transferred to a 10-ml screw-capped centrifuge tube and evaporated to dryness under air at 50°. This constituted the basic extract. The remaining ether in the original extraction tube was carefully removed with a Pasteur pipette and discarded. Sulfuric acid (5 M, 0.5 ml) was added to the urine aqueous phase. This acidified urine layer was extracted with 8 ml of anhydrous ether and centrifuged as described above for the basic extract. A 2-ml aliquot of the ether layer was transferred to a 10-ml centrifuge tube and evaporated to dryness yielding the acid extract. Both the acidic and basic extracts were reconstituted in mobile phase (100 μl) prior to injection of a 20-μl aliquot into the high-pressure liquid chromatograph.

For the basic extracts an acetonitrile—methanol—acetic acid—water (35:5:1:59) mobile phase was prepared and degassed for 2 h before use. A mobile phase consisting of acetonitrile—acetic acid—water (36:1:63) was prepared, degassed and employed in the analysis of all acid extracts. Flow-rate for both separations was 2.0 ml/min with the fluorescence and absorbance detector responses being recorded simultaneously on a dual channel strip chart recorder.

Deconjugation of urinary propranolol metabolites

To 1.0 ml of urine contained in a 15-ml screw-capped centrifuge tube was added 0.2 ml of 1 M acetate buffer (pH 4.7), 100 μl of 20% sodium metabisulfite, and 30 μl of a deconjugating enzyme solution yielding a final concentration of 0.20 I.U./ml β-glucuronidase and 0.25 I.U./ml aryl sulfatase (*Helix promatia*; Calbiochem, La Jolla, Calif., U.S.A.) The reaction mixture was capped and placed in a water bath at 45° for 4 h. Following the incubation, the acetate buffer was neutralized by the addition of 0.2 ml of 1 M NaOH. The deconjugated urine sample was then subjected to the assay procedure described above.

Preparation of standard curves

A stock solution of standards in methanol was prepared containing 53 μg/ml propranolol glycol, 60 μg/ml N-desisopropylpropranolol, 80 μg/ml α-naphthol, 50 μg/ml naphthoxyacetic acid, 520 μg/ml naphthoxylactic acid, and 492 μg/ml propranolol hydrochloride. A separate stock solution containing 471 μg/ml 4-hydroxypropranolol hydrochloride was also prepared. Standard solutions were prepared from each stock solution as follows: standard No. 1, 1:20 dilution of stock; standard No. 2, 1:10 dilution; standard No. 3, 1:4 dilution; standard No 4, 1:2 dilution; standard No. 5, straight stock solution. Standard curves were prepared by addition of 100 μl of each standard solution to the appropriate 15-ml screw-capped centrifuge tube and evaporating the methanol to dryness. One ml of drug-free urine was then added to each tube and the standards extracted and assayed as described above. Equations for the standard curves were calculated from the data by simple linear regression. All standard solutions in methanol were stored at 4° between use. The 4-hydroxypropranolol hydrochloride standards were freshly made up each week. New standard solutions containing propranolol hydrochloride and the rest of the metabolites were prepared monthly.

Stability of 4-hydroxypropranolol

In order to assess the stability of the 4-hydroxypropranolol standard solutions in methanol, four injections (10 μl) of a fresh solution of 4-hydroxypropranolol in methanol (15.2 μg/ml) were made into the chromatograph and the resultant peak heights recorded. This procedure was repeated 1,2,5, and 8 days later using the same solution of 4-hydroxypropranolol. Between injections the 4-hydroxypropranolol solution in methanol was stored at 4°.

Experiments were performed to assess the effect of various anti-oxidants on the stability of 4-hydroxypropranolol in urine. A 50-ml volume of freshly collected urine (drug free) was spiked with 168 μg of 4-hydroxypropranolol hydrochloride and 47 μg of propranolol hydrochloride. Sets of 8, 15-ml screw-capped centrifuge tubes, each containing 1 ml of the spiked urine, were treated by addition of one of the following reducing agents; no reducing agent; 100 μl, 20% sodium metabisulfite; 100 μl, 20% sodium bisulfite; 100 μl, 20% sodium dithionite and 50 μl, 10% sodium ascorbate. Four of the tubes in each group were stored for seven days at room temperature under nitrogen; the remaining four tubes were stored under air. All samples were then extracted, assayed and the peak height ratio of 4-hydroxypropranolol vs. propranolol recorded. These

ratios were then compared with the ratios obtained from freshly extracted spiked urine samples. Propranolol is stable under these conditions.

Stability of propranolol metabolites in frozen urine

Drug-free urine (100 ml) was spiked with the following compounds: 94 μg propranolol HCl; 98 μg N-desisopropylpropranolol; 152 μg 4-hydroxypropranolol HCl; 79 μg propranolol glycol; 78 μg naphthoxyacetic acid; and 228 μg naphthoxylactic acid. Groups of 6 screw-capped centrifuge tubes, each containing 1 ml of spiked urine, were frozen and assayed after 0,1,5, or 8 days of storage. Three of the 6 samples in each group also contained 100 μl of 20% sodium metabisulfite during storage. The peak height ratios of 4-hydroxypropranolol, propranolol glycol and N-desisopropylpropranolol to propranolol were determined at each time period. In addition, the peak height ratio of naphthoxylactic acid vs. naphthoxyacetic acid as assayed from the acid extracts were recorded.

Stability of propranolol and metabolites following extraction

Experiments were conducted in which the stability of 4-hydroxypropranolol and other metabolites were assessed following three post-extraction storage treatments. The first treatment involved dissolving the evaporated ether extract in mobile phase and storing it overnight at room temperature prior to injection into the chromatograph. The second storage procedure consisted of leaving the samples in the evaporated state at room temperature overnight prior to reconstitution in mobile phase and injection onto the chromatograph. A third treatment involved dissolution of evaporated extracts in methanol, storage overnight at 4°, evaporation to dryness under nitrogen and subsequent reconstitution in mobile phase and injection onto the chromatograph. These storage procedures were performed on 5—10 urine samples spiked with standard No. 3. The concentration of propranolol and the metabolites measured following each pretreatment was determined and compared to the values obtained from freshly extracted samples.

Stability of propranolol and its metabolites during deconjugation

Six 1-ml aliquots of urine, spiked with standard No. 3, were subjected to the deconjugation described above. Following 4 h of incubation, the concentrations of propranolol and metabolites were determined and compared to the initial amounts present prior to incubation.

RESULTS AND DISCUSSION

The HPLC separation of basic extracts from urine containing propranolol, 4-hydroxypropranolol, N-desisopropylpropranolol, α-naphthol, propranolol glycol and the internal standard, procainamide, is visualized in the absorbance and fluorescence chromatograms of Fig. 2. A mobile phase consisting of acetonitrile—methanol—acetic acid (35:5:1) was found to provide a good separation of propranolol and its metabolites without interference by other urinary constituents. Although fluorescence detection adds greater sensitivity to the analysis, it can be seen that absorbance responses at 295 nm would also provide
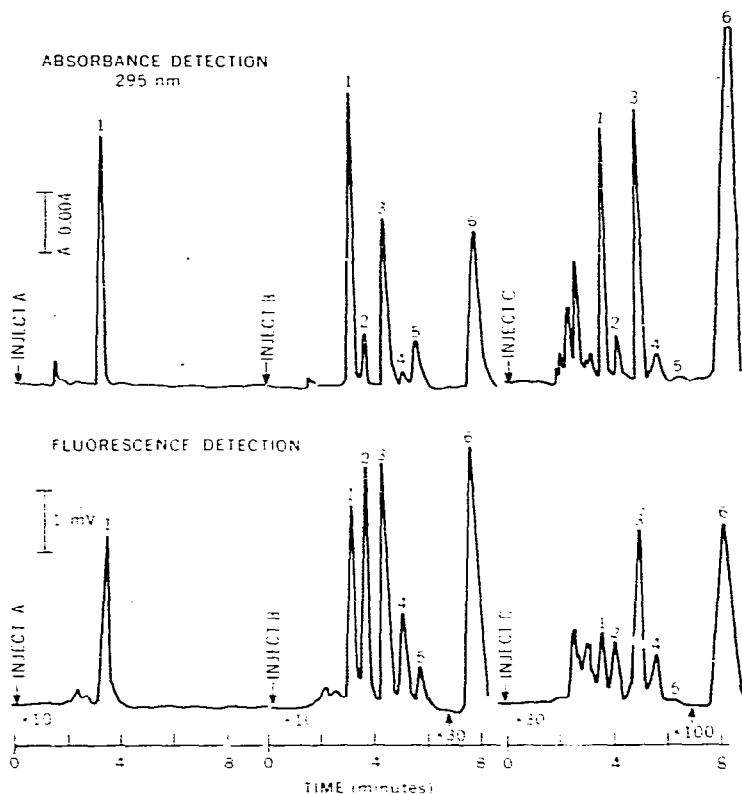
Fig. 2. The absorbance and fluorescence chromatograms of basic extracts from human urine. 1=Procainamide; 2=propranolol glycol; 3=4-hydroxypropranolol; 4=N-desisopropylpropranolol; 5=α-naphthol; 6=propranolol. A = Drug free urine; B = spiked urine, standard No. 3; C = deconjugated urine from a patient treated with propranolol, orally.

an adequate assay for many of these compounds in human urine, particularly propranolol and 4-hydroxypropranolol. A major advantage of recording both fluorescence and absorbance responses simultaneously is that, should unexpected background peaks appear in the fluorescence chromatograms of patient urine samples, the absorbance response often remains unchanged and thus can be used as an alternative method of analysis.

Of many compounds tested, procainamide emerged as the most suitable candidate as an internal standard. It is efficiently extracted under the basic conditions of the assay, has fluorescence and absorbance qualities comparable to propranolol and its metabolites, and is adequately resolved from background urine peaks and those of propranolol and its metabolites under the chromatographic conditions employed. However, urines from patients receiving both propranolol and procainamide cannot be analyzed by this methodology utilizing procainamide as the internal standard. The search continues for non-drug internal standards which may be applied to this analysis.

Fig. 3 depicts the absorbance and fluorescence chromatograms of the acid extracts. In this case fluorescence detection provides a much cleaner as well as a more sensitive method of detection for naphthoxylactic and naphthoxyacetic

ABSORBANCE DETECTION
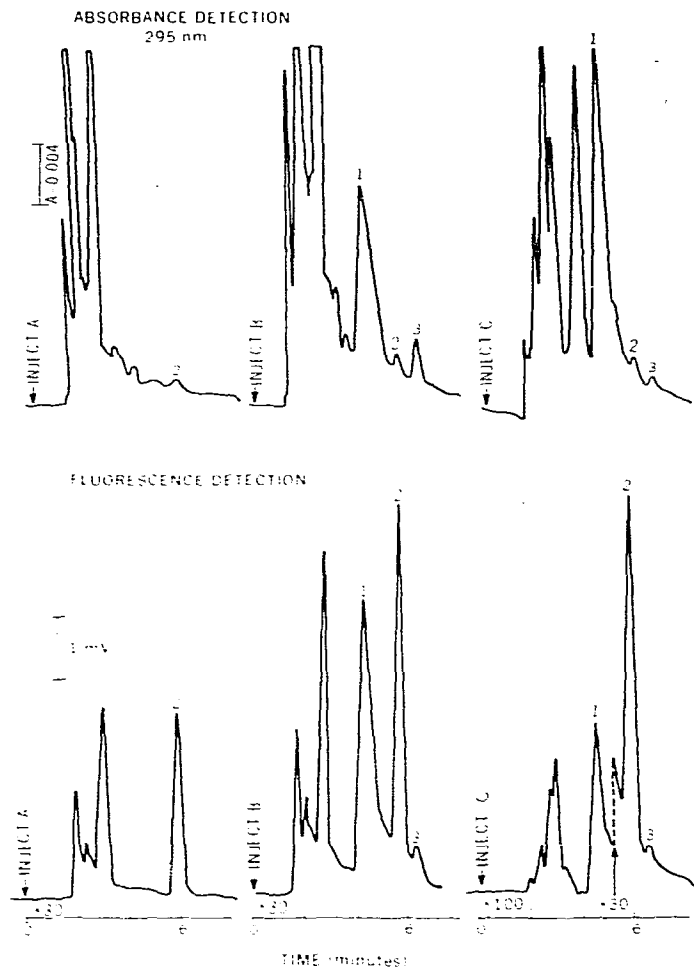295 nm

FLUORESCENCE DETECTION

TIME (minutes)

Fig. 3. The absorbance and fluorescence chromatograms of acidic extracts from human urine. 1=1-naphthoxylactic acid, 2=2-naphthoxyacetic acid, 3=1-naphthoxyacetic acid. A = Drug free urine; B = spiked urine, standard No. 3; C = urine from patient treated with propranolol orally.

acids than absorbance monitoring. 2-Naphthoxyacetic acid, a structural isomer of the metabolite, 1-naphthoxyacetic acid, had excellent fluorescent properties and proved to be a suitable internal standard.

Extraction of the urines with ether yielded much cleaner extracts than solvents such as ethyl acetate and benzene which have been employed in previous methods for the analysis of propranolol and/or its metabolites [9—11]. A pH between 9.5 and 10.0 was essential to ensure efficient extraction of 4-hydroxy-propranolol [10]. Addition of 100 μl of the antioxidant, 20% sodium metabisulfite, reduced the urine pH by about 0.4 units in the presence of 1 M carbonate buffer. Therefore, it was necessary to employ a carbonate buffer whose pH was slightly greater than 10 (10.2) to yield a final extraction pH of between 9.5 and 10. Under acidic conditions, significant amounts of propranolol and the other basic metabolites may be extracted and interfere with the analysis of

the acid metabolites [3]. Therefore, it was necessary to perform the basic extraction step first. Under the conditions of the basic extraction, none of the acidic metabolites were extracted.

Excellent linearity ($r > 0.990$) was observed for the standard curves in all cases with the exception of α-naphthol. The coefficient of variation (C.V.) for α-naphthol was 14.7% (Table I). Since α-naphthol was found to be a minor urinary metabolite of propranolol (Table IV) further efforts to enhance the precision of the α-naphthol measurements were not pursued. The coefficients of variation for propranolol and the other basic metabolites were all less than 5% whereas naphthoxylactic acid and naphthoxyacetic acid yielded C.V.'s of 6.3 and 6.0% respectively (Table I).

TABLE I

INTRA-ASSAY VARIATION OF PROPRANOLOL AND ITS METABOLITES

C.V.'s were determined from the assay of 10 urine samples spiked with the concentrations of each compound indicated.

	Concentration (µg/ml)	C.V.
Propranolol	12.3	2.3
4-Hydroxypropranolol	11.8	5.0
N-Desisopropylpropranolol	1.5	3.6
Propranolol glycol	1.3	1.7
α-Naphthol	2.0	14.7
Naphthoxylactic acid	13.0	6.3
Naphthoxyacetic acid	1.25	6.0

Daily variations in the slope of the standard curves do occur making it necessary to run standards with each set of unknown samples. In a three week period during which five, five-point standard curves were constructed, the slopes ranged for each compound as follows: propranolol glycol, 0.870 to 0.959; 4-hydroxypropranolol hydrochloride, 0.0759 to 0.0992; N-desisopropylpropranolol, 0.265 to 0.322; α-naphthol, 0.090 to 0.145; propranolol hydrochloride, 0.301 to 0.344; naphthoxylactic acid, 0.0305 to 0.0359; and naphthoxyacetic acid, 0.0216 to 0.0274.

Recently Nation et al. [12] have described a HPLC method for the analysis of propranolol and 4-hydroxypropranolol in plasma. A comparison with our analysis for these compounds from urine reveals similar assay conditions. We have selected ether rather than ethyl acetate as the extracting solvent since in our experience less contamination by undesirable urinary compounds has been obtained. The excitation wavelength employed in the urine assay was 295 nm instead of 205 nm used by the above workers in plasma. Krol et al. [13] have demonstrated that the relative fluorescence of propranolol and 4-hydroxypropranolol is greater at excitation wavelengths less than 250 nm as compared to wavelengths greater than 250 nm. However, the sensitivity achieved with the 295 nm excitation wavelength is more than adequate to quantitate free and

conjugated forms of propranolol and its other basic metabolites in human urine.

Previous attempts to measure the 4-hydroxylated metabolite of propranolol in plasma have been hampered by the instability of this molecule. Walle et al. [10] stated that 4-hydroxypropranolol was stable for 7 h in methanol. In the present study, 1 day of storage in methanol at 4° yielded 104±3% of the peak height of 4-hydroxypropranolol when freshly prepared; 2 days storage, 97±1%; 5 days storage, 99±1%; and 8 days storage, 92±1%. Therefore, 4-hydroxypropranolol standard solutions in methanol were stable for up to one week if stored refrigerated between use.

Previous studies stated that 4-hydroxypropranolol was stable in plasma if a 2% solution of the antioxidant, sodium bisulfite, was present [10]. Experiments were conducted to establish the relative stability of 4-hydroxypropranolol in urine in the presence of various antioxidants (Table II). When no reducing agent was employed, virtually all the 4-hydroxypropranolol initially present in the urine samples was lost after 7 days of storage at room temperature. A 2% solution of sodium metabisulfite or sodium bisulfite completely protected 4-hydroxypropranolol from oxidation. Sodium dithionite and sodium ascorbate were less effective antioxidants. A nitrogen atmosphere yielded little added stability. In view of the efficacy of sodium metabisulfite in preserving 4-hydroxypropranolol in urine it was selected as the antioxidant for the assay procedure.

Freezing of fresh urine samples and storage for up to 8 days, either with or without 2% sodium metabisulfite, resulted in no significant changes in the levels of 4-hydroxypropranolol, N-desisopropylpropranolol, and propranolol glycol present relative to propranolol which itself is stable under such conditions. Naphthoxylactic acid levels in the urine were also stable over the 8 day study period when frozen upon collection. These results agree with previous studies [10] which reported that 4-hydroxypropranolol was stable in plasma for up to 1 week if frozen.

The stability of propranolol and its metabolites after extraction was also

TABLE II

STABILITY OF 4-HYDROXYPROPRANOLOL IN THE PRESENCE OF VARIOUS ANTI-OXIDANTS

Samples were stored at room temperature for 7 days either directly exposed to air or capped under nitrogen. Results are expressed as the percentage of 4-hydroxypropranolol peak height ratio vs. propranolol relative to that measured in fresh extracts (100±3). Values represent the mean ± S.E. for 4 determinations. Initial 4-hydroxypropranolol concentration was 8.2 μg/ml urine.

	Air	Nitrogen
No reducing agent	3 ± 1	21 ± 17
2% Sodium metabisulfite	97 ± 3	95 ± 6
2% Sodium bisulfite	96 ± 3	96 ± 5
2% Sodium dithionite	65 ± 14	91 ± 3
0.5% Sodium ascorbate	72 ± 8	60 ± 7

assessed. 4-Hydroxypropranolol was unstable if left overnight in mobile phase ($45\pm5\%$ of initial concentration) or in the evaporated state ($89\pm2\%$). In addition, only $76\pm3\%$ and $80\pm3\%$ of the added naphthoxylactic and naphthoxyacetic acid was recovered following storage overnight after evaporation. Prolonged exposure of the acid metabolites to the 5 M sulfuric acid (required for efficient extraction) may result in significant hydrolysis of these compounds. Thus, the shaking time should not exceed 15 min and storage in methanol following extraction should not exceed 2 h. Stability of all of the other metabolites with the exception of α-naphthol ($45\pm9\%$ reduction in activity) was preserved if the samples were reconstituted in methanol and stored at $4°$. However, best results were obtained if: (1) samples were extracted and injected onto the chromatograph the same day; (2) samples were not left reconstituted in mobile phase any longer than 2 h prior to analysis.

Previous studies have suggested that propranolol and its basic metabolites are excreted in the urine predominantly as their glucuronide and/or sulfate conjugates [3,4;14]. Preliminary studies on hypertensive patients receiving chronic propranolol therapy appear to substantiate these observations (Table IV). Therefore, if estimates of total amounts of propranolol and metabolites (free plus conjugated) are desired it is necessary to deconjugate urine samples prior to analysis. Under the conditions of the deconjugation reaction, maximum release of conjugated metabolites was achieved following 4 h of incubation. All the basic metabolites of propranolol with the exception of α-naphthol were stable during deconjugation in the presence of 2% sodium metabisulfite (Table III). The reducing agent had no effect on the deconjugating enzyme preparation. The acidic metabolites were also stable under these conditions.

Propranolol and its metabolites were determined in 24-h urine collections from 3 patients chronically treated with 40 mg propranolol, 4 times daily (Table IV). All the basic compounds existed predominantly as O-glucuronides

TABLE III

STABILITY OF PROPRANOLOL AND ITS METABOLITES DURING THE DECONJUGATION REACTION

Results are expressed as the percentage of the initial concentration remaining after 4 h of incubation at $37°$ with β-glucuronidase—aryl sulfatase at pH 4.7. Values represent the mean ± S.E. of 6 determinations. The incubation was carried out in the presence of 2% sodium metabisulfite.

	Initial concentration (μg/ml)	Percent recovery after incubation
Propranolol	12.3	99 ± 2
4-Hydroxypropranolol	11.8	98 ± 2
N-Desisopropylpropranolol	1.5	99 ± 5
Propranolol glycol	1.3	102 ± 3
α-Naphthol	2.0	66 ± 11
Naphthoxylactic acid	13.0	112 ± 2
Naphthoxyacetic acid	1.25	110 ± 4

TABLE IV

24-H URINARY EXCRETION DATA FROM THREE PATIENTS CHRONICALLY TREATED WITH PROPRANOLOL

Each patient was receiving 40 mg propranolol hydrochloride, 4 times daily (542 μmoles). Sodium metabisulfite (20 g) was added to each urine bottle prior to collection. 1,2,3 = Patient numbers.

	Total μmoles excreted			Percent of dose			Percent conjugated		
	1	2	3	1	2	3	1	2	3
Propranolol	87.5	58.4	44.4	16.1	10.8	8.2	91	98	82
4-Hydroxypropranolol	13.6	25.4	27.7	2.5	4.7	5.1	93	87	88
Naphthoxylactic acid	90.1	152.6	106.9	16.6	28.2	19.7	0	0	0
N-Desisopropylpropranolol	2.8	0.9	7.9	0.5	0.2	1.5	92	76	100
Propranolol glycol	6.9	7.9	6.5	1.3	1.5	1.2	100	100	100
α-Naphthol	6.1	13.5	2.7	1.1	2.5	0.5	100	100	100
Naphthoxyacetic acid	nil	nil	nil	nil	nil	nil	–	–	–
Total	207.1	258.7	196.1	38.1	47.9	36.2			

or sulfated conjugates which could be liberated by the β-glucuronidase—aryl sulfatase enzyme preparation. Attempts were made to free any N-glucuronide conjugates present through acid hydrolysis [15] of patients urines in 0.2 M HCl for 15 min at 25°. No significant levels of propranolol and its metabolites were recorded above the concentrations of free propranolol and metabolites already present in the urines suggesting that N-glucuronidation does not play a significant role in the metabolic disposition of propranolol or the metabolites studied.

The major recoverable urinary excretion products were 4-hydroxypropranolol, naphthoxylactic acid and propranolol. These compounds have previously been identified as major urinary excretion products of propranolol in man [2—4] although the quantitative contribution of each compound to the metabolic disposition of propranolol is unknown. Early studies using ^{14}C-labelled propranolol [2] indicated that 85—95% of a single 40 mg dose in man was eventually excreted in the urine. Of interest was the finding in the present study that 60% of the administered dose remained unaccounted for in the patients studied suggesting that other unmeasured metabolites are present in these urines. Further studies are in progress to define the quantitative profile of propranolol metabolism in man.

ADDENDUM

Further experience with the method described has shown that: (1) different columns might have different chromatographic properties. Minor adjustment in the composition of the mobile phase can correct for this discrepancy and allow for appropriate resolution of the compounds measured; (2) 1-naphthalene-ethanol (Aldrich) has been found to be a suitable non-drug internal standard.

A mobile phase acetonitrile—methanol—acetic acid—water (35:20:0.075:45), pH 3.4, has been used to obtain an adequate separation of propranolol and its metabolites using this internal standard; (3) absorbance detection at 310 nm has been found to provide sensitive measurement of 4-hydroxypropranolol free of any background which may be present in deconjugated urine.

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