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RAPID AND SIMULTANEOUS EXTRACTION OF PROPRANOLOL, ITS NEUTRAL AND BASIC METABOLITES FROM PLASMA AND ASSAY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of propranolol, its neutral and basic metabolites from a single plasma sample. These analytes were extracted simply and efficiently by a solid-phase extraction column based on C_{18} modified silica (C_{18} Bond-Elut™). Propranolol, the 4-hydroxy and N-desisopropyl metabolites were separated on a μBondapak C_{18} column with a mobile phase of acetonitrile—0.1% phosphoric acid. Propranolol glycol was selectively eluted from the C_{18} Bond-Elut column with acetonitrile and chromatographed separately with a mobile phase of acetonitrile—water. The recoveries of propranolol and all metabolites were greater than 78% with an intra-assay coefficient of variation between 4.9 and 7.3% at a concentration of 5—50 ng/ml. The minimum detectable levels in 1 ml of plasma were 1.0 ng/ml propranolol, 6.0 ng/ml 4-hydroxypropranolol, 1.0 ng/ml N-desisopropylpropranolol and 2.5 ng/ml propranolol glycol.

Enzyme hydrolysis, Bond-Elut extraction and high-performance liquid chromatography revealed that propranolol, the neutral and basic metabolites were extensively conjugated in dog plasma (propranolol 67%, 4-hydroxypropranolol 98%, N-desisopropylpropranolol 55% and propranolol glycol 80%). With the use of pure enzymes and a selective inhibitor the nature of this conjugation appeared to involve both glucuronidation and sulfation. The conjugation of propranolol involved mainly glucuronidation (58—62%) compared to sulfation (7—12%), whilst that of 4-hydroxypropranolol mainly involved sulfation (55—65%) compared to glucuronidation (32—38%). The values for N-desisopropylpropranolol and propranolol glycol were 26—31% and 12% sulfation, 16—29% and 68—85% glucuronidation, respectively.

INTRODUCTION

The disposition and pharmacokinetics of propranolol, a β-adrenergic receptor

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antagonist which is extensively metabolised in rat, dog and man [1], has been the subject of intensive investigation for many years. Large variations in the plasma levels of propranolol and its active metabolites [2—5], has led to the development of many analytical techniques in an effort to optimize therapy and to correlate pharmacological activity with drug levels.

The techniques that have been employed are: spectrofluorimetry [6—11], gas chromatography (GC) [12—17] and high-performance liquid chromatography (HPLC) [18—33]. The spectrofluorimetric methods have been used mainly to assay propranolol itself. The disadvantages of poor sensitivity and questionable specificity [16, 17, 34] make the methods unsuitable for the assay of propranolol metabolites.

These disadvantages were overcome by the GC methods, however, these required sophisticated instrumentation (e.g. mass spectrometry or electron-capture detection) and required lengthy derivatization steps. Most methods reported recently have been based on HPLC which gives both high selectivity and sensitivity without the need for derivatization.

Extraction of drugs and metabolites from biological samples remains the main difficulty in drug analysis. In the case of propranolol and its metabolites this is usually achieved by solvent extraction from alkalinized plasma or urine [12—32]. The extraction procedures can be lengthy and tedious often resulting in poor recoveries of metabolites sensitive to alkali such as the 4-hydroxypropranolol [23, 35]. An alternative to solvent extraction is protein precipitation with acetonitrile [33] which has the advantage of being quick and convenient but lacks the selectivity and sensitivity for the analysis of propranolol metabolites [31].

We report a new extraction method based on the use of short columns of reversed-phase silica (C_{18} Bond-Elut^{TM}) which quantitatively extracts propranolol, 4-hydroxypropranolol, N-desisopropylpropranolol and propranolol glycol without the need for alkalinization of plasma. The method is rapid, technically simple and coupled with standard HPLC procedures leads to a sensitive, accurate and reproducible assay for propranolol and its major metabolites. The basic extraction method has recently been applied successfully to the assay of atenolol and metoprolol [36] and baclofen [37].

EXPERIMENTAL

Materials and reagents

Propranolol · HCl, 4-hydroxypropranolol · HCl, N-desisopropylpropranolol · HCl and propranolol glycol · HCl were kindly supplied by ICI (Macclesfield, U.K.). Acetonitrile (HPLC grade) was supplied by Waters Assoc. (Sydney, Australia). All other reagents were of analytical grade. Bond-Elut (1 ml, C_{18} silica) columns and Vac-Elut chamber were obtained from FSE Scientific (Melbourne, Australia).

Instrumentation

The HPLC system (system 1) consisted of an LDC Constametric III pump (LDC, Riviera Beach, FL, U.S.A.), a Schoeffel FS970 fluorescence detector (Schoeffel Instruments, Westwood, NJ, U.S.A.) with excitation wavelength set

at 217 nm and a 360-nm cut-off filter (Schoeffel) for emission, a Rheodyne 7010/7011 injection valve with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), a 30 cm × 4.0 mm I.D., 10 μm, C_{18} μBondapak column (Waters Assoc.) and an Omniscribe chart recorder (Houston Instruments, Austin, TX, U.S.A.).

A similar HPLC system (sytem 2) was used to measure propranolol glycol, consisting of a Varian Fluorichrom fluorescence detector (Varian Assoc., CA, U.S.A.) fitted with a 220-nm interference filter for excitation and a 7-60 cut-off filter (Varian) for emission, and a Rheodyne 3176 injection valve.

Preparation of standards

Stock solutions containing 1 mg/ml propranolol · HCl or metabolite were made in distilled water (basic metabolites) or methanol (propranolol glycol) and stored at 4°C. From these solutions a stock plasma standard was made containing 10 μg/ml propranolol · HCl, 5 μg/ml N-desisopropylpropranolol · HCl, 20 μg/ml 4-hydroxypropranolol · HCl and 5 μg/ml propranolol glycol · HCl and stored frozen. The stock plasma standard was further diluted with drug-free plasma to give the final plasma standards which were stored frozen at −20°C. A fresh stock plasma standard was made each week from stock aqueous (or methanol) solutions and in the case of 4-hydroxypropranolol a fresh stock aqueous solution was also made.

Extraction procedure

C_{18} Bond-Elut columns were placed in luer fittings in the top of the Vac-Elut™ cover, which has the capacity for ten columns. A vacuum of 25—50 cmHg was applied to the manifold to effect the various stages of the extraction procedure. The columns were activated before use by washing with 1-ml aliquots of 0.1 *M* hydrochloric acid, twice, 1 ml of acetonitrile, twice, followed by 1 ml of distilled water, twice. Plasma (1 ml sample or standard) was then passed through the column followed by 0.5 ml of distilled water—acetonitrile (90:10), twice. The vacuum was then released and the stainless-steel needles of the Vac-Elut lid wiped. Appropriately labelled polypropylene tubes were placed under the columns which were then eluted with two 0.5-ml aliquots of acetonitrile under vacuum. After collecting the acetonitrile eluent (containing propranolol glycol), the vacuum was released and fresh collecting tubes were placed under the columns to which was added 0.5 ml acetonitrile—0.1 *M* hydrochloric acid (50:50) and the vacuum re-applied. This second wash eluted the propranolol and its basic metabolites from the Bond-Elut column. Samples (20 μl) of the collected eluents were then injected onto the appropriate HPLC column. The same Bond-Elut column can be used several times by following the preliminary washing procedures outlined above so that a set of standards and samples can all be run on the column.

Enzyme hydrolysis

To plasma (0.5 ml, sample or standard) in small plastic tubes was added 0.1 ml of 1 *M* sodium acetate solution pH 4.8, containing 20 mg/ml ascorbic acid as an antioxidant, followed by 0.1 ml of enzyme solution containing 10 000 U/ml β-glucuronidase per 370 U/ml sulfatase (Sigma G 0876). After

vortexing, the tubes were placed in a water bath at 37°C for 1 h. After incubation 0.1 *M* sodium hydroxide (0.2 ml) was added to the tubes which were then vortexed and centrifuged for 5 min at 1600 *g*. A 0.7-ml aliquot of the supernatant was then extracted as described above.

High-performance liquid chromatography

Propranolol and its basic metabolites were separated on a 10-μm C_{18} μBondapak column with a mobile phase of acetonitrile—0.1% orthophosphoric acid (23:77) at a flow-rate of 2 ml/min (system 1). The Bond-Elut columns extracted most of the major propranolol metabolites (basic and acidic), however, the resolution of these with one chromatographic system proved difficult. For example, with the acetonitrile—phosphoric acid mobile phase propranolol glycol and naphthoxylactic acid metabolites co-elute at a retention time of 13 min, while a mobile phase of acetonitrile—distilled water—glacial acetic acid (30:69.8:0.2) separated the naphthoxylactic acid from the propranolol glycol, but failed to resolve 4-hydroxypropranolol and N-desisopropylpropranolol from other extracted, but unidentified, minor propranolol metabolites. However, it was found that by eluting the Bond-Elut column with acetonitrile, propranolol glycol and some of the naphthoxylactic acid could be selectively eluted, without major losses of propranolol or its basic metabolites. The propranolol glycol and naphthoxylactic acid metabolites in the acetonitrile extract could then be separated on a 10-μm, C_{18} μBondapak column using a mobile phase of acetonitrile—distilled water (30:70) at a flow-rate of 2 ml/min (system 2). Under these conditions propranolol glycol eluted after 8.1 min and naphthoxylactic acid after 1.0 min.

The concentration of propranolol and its metabolites was determined by extracting plasma standards under the same conditions and measuring peak heights to give calibration plots.

Recovery

The recovery of propranolol and its metabolites was estimated by measuring peak heights of non-extracted solutions in the appropriate Bond-Elut elution solvent and comparing the results obtained from extracting plasma standards of the same concentration.

RESULTS AND DISCUSSION

Chromatograms, before and after enzyme hydrolysis, of drug-free dog plasma and plasma from the post-hepatic vein of a propranolol-dosed dog (propranolol infusion into the jejunum at 11 μg/kg/min) are shown in Fig. 1 for propranolol and basic metabolites (system 1) and Fig. 2 for propranolol glycol (system 2).

The total assay time for propranolol and its basic metabolites was approximately 15 min (system 1), whilst for propranolol glycol (system 2) it was approximately 10 min, with 1—2 min extraction time. The speed and ease of this method along with the high extraction efficiencies for propranolol and its metabolites, without the need for high pH, gives the method its accuracy and good reproducibility as shown by the data given in Table I.

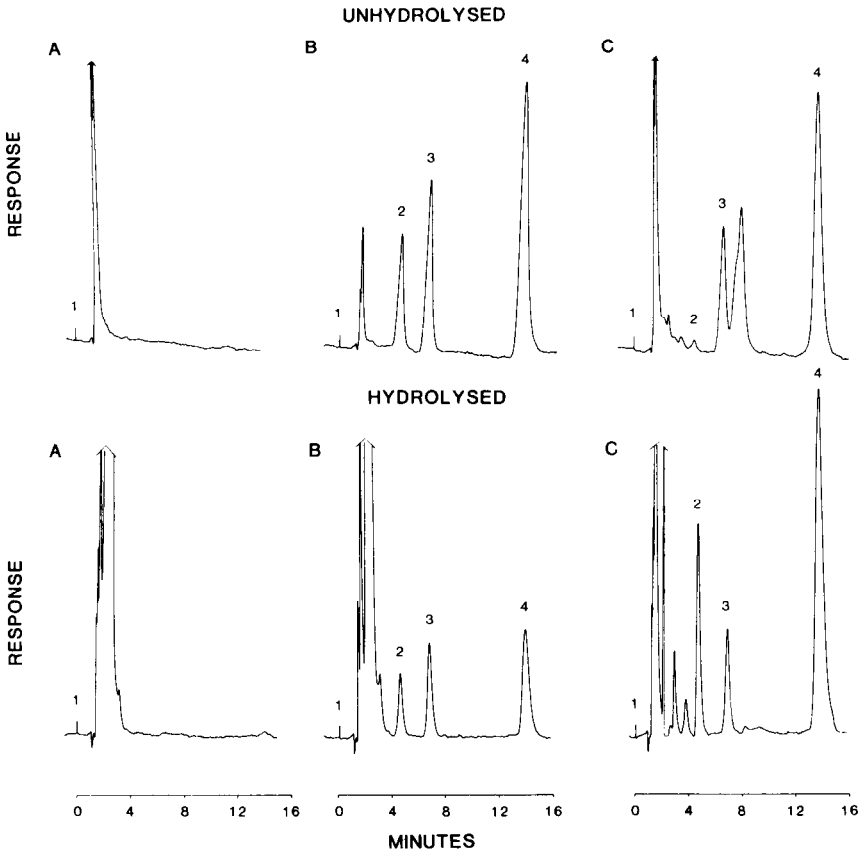

Fig. 1. Separation of propranolol and metabolites before and after hydrolysis with β-glucuronidase/sulfatase for 1 h at 37°C. Mobile phase: acetonitrile—0.1% orthophosphoric acid (23:77); flow-rate 2 ml/min. (A) Extracted drug-free plasma; (B) extracted drug-free plasma containing 200 ng/ml 4-hydroxypropranolol · HCl (2), 50 ng/ml N-desisopropylpropranolol · HCl (3) and 100 ng/ml propranolol · HCl (4); (C) extracted plasma from post-hepatic vein of dog infused with propranolol. 1 = Sample injection.

The recovery of propranolol from plasma with this method was high and compares well with the recoveries of propranolol obtained with solvent extraction methods [9, 10, 24, 32, 35]. Good recovery of all the metabolites with solvent extraction is difficult due to their differential extraction efficiencies. However, Lo et al. [31] reported similar recoveries to ours for 4-hydroxypropranolol (68—81%) and N-desisopropylpropranolol (59—82%) extracting alkalinized plasma with diethyl ether followed by an acid back-extraction.

The long-term stability of the method is reflected by the low coefficient of variation (C.V.) for the slopes of the calibration lines over a period of nine months, 6.9% (n = 18) propranolol, 12.1% (n = 18) 4-hydroxypropranolol and 7.9% (n = 18) N-desisopropylpropranolol.

Propranolol, the neutral and basic metabolites have been reported to be extensively conjugated in urine from human volunteers [23]. In plasma, propranolol and 4-hydroxypropranolol are 75% and 95% conjugated, respectively, whilst the N-desisopropylpropranolol is largely unconjugated

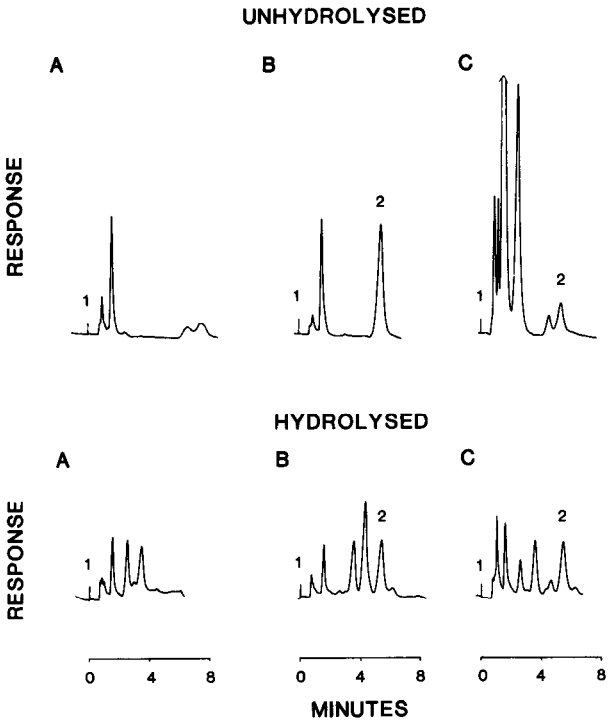

Fig. 2. Separation of naphthoxylactic acid and propranolol glycol before and after hydrolysis with β-glucuronidase/sulfatase for 1 h at 37°C. Mobile phase: acetonitrile—distilled water (30:70); flow-rate 2 ml/min. (A) Extracted drug-free plasma; (B) extracted drug-free plasma containing 50 ng/ml propranolol glycol · HCl (2); (C) extracted plasma from post-hepatic vein of dog infused with propranolol. 1 = Sample injection.

TABLE I

RECOVERIES AND INTRA-ASSAY VARIATION OF PROPRANOLOL AND ITS METABOLITES IN PLASMA

Compound	Concentration (ng/ml)	Percentage recovery (mean ± S.D., n = 7)	Intra-assay C.V. (n = 7) (%)
Propranolol	500	86.7 ± 1.6	1.9
	100	87.6 ± 3.4	3.9
	10	79.5 ± 3.9	4.9
4-Hydroxypropranolol	1000	89.9 ± 1.9	2.1
	200	86.3 ± 5.2	6.0
	20	92.4 ± 6.0	6.4
N-Desisopropylpropranolol	250	81.1 ± 2.9	3.6
	50	79.0 ± 3.3	4.2
	5	78.1 ± 5.7	7.3
Propranolol glycol	250	93.1 ± 1.4	1.5
	50	87.8 ± 4.3	4.9

[31]. To prevent the oxidation of 4-hydroxypropranolol during enzyme hydrolysis antioxidants such as sodium metabisulfite [25, 28, 33], sodium bisulfite [12—14, 25—30] and ascorbic acid [25, 31, 33] have been included

TABLE II

HYDROLYSIS OF CONJUGATES WITH AND WITHOUT ASCORBIC ACID

Compound	Concentration (ng/ml)			
	Incubation time (h)			
	0	0.5	1.0	1.5
Propranolol				
Without ascorbic acid	82.6	257.5	237.9	246.4
With ascorbic acid	—	251.8	253.0	247.7
4-Hydroxypropranolol				
Without ascorbic acid	17.5	613.4	460.1	388.4
With ascorbic acid	—	701.7	738.9	743.9
N-Desisopropylpropranolol				
Without ascorbic acid	18.0	35.8	34.6	40.9
With ascorbic acid	—	34.2	40.2	37.5
Propranolol glycol				
Without ascorbic acid	8.4	42.3	44.9	43.1
With ascorbic acid	—	41.6	41.2	41.0

TABLE III

NATURE OF CONJUGATION OF PROPRANOLOL AND ITS METABOLITES

(A) Enzyme: sulfatase; incubation time: 1 h. (B) Enzyme: sulfatase; incubation time: 2 h. (C) Apparent percentage sulfation observed with the mixed enzyme inhibited with D-glucaro-1,4-lactone. (D) Apparent sulfation determined by the difference between total percent conjugates (uninhibited mixed enzyme), the percent glucuronidation (determined with pure glucuronidase) and the percent unconjugated.

Compound	Percentage unconjugated	Percentage sulfated				Percentage glucuronidated*
		A	B	C	D	
Propranolol	32.6	8.0	6.7	11.9	5.6	61.8
4-Hydroxy-propranolol	2.4	32.3	54.9	65.0	65.2	32.4
N-Desisopropyl-propranolol	44.8	27.6	21.4	31.3	39.0	16.2
Propranolol glycol	20.4	12.0	12.0	12.0	0.0	85.0

*Enzyme: β-glucuronidase; incubation time: 1 h.

in the incubation. We found that the inclusion of ascorbic acid at 0.2 mg/ml and incubation with 1000 U β-glucuronidase per 37 U sulfatase at 37°C for 1 h released the conjugates of all the metabolites studied with no significant losses of the 4-hydroxy metabolite (Table II). Ascorbic acid was preferable to bisulfite (most commonly used) as the latter has been shown to inhibit the sulfatase component of the mixed enzyme [38]. The results in Table II show that in dog plasma propranolol, the basic and the neutral metabolites are extensively conjugated.

The intra-assay coefficient of variation after hydrolysis of several samples

(n = 7) of the plasma from a propranolol-dosed dog; 2.9% propranolol, 3.7% 4-hydroxypropranolol, 3.4% N-desisopropylpropranolol and 5.0% for propranolol glycol, are comparable with those obtained earlier for the non-incubated samples (Table I).

The nature of the conjugation of propranolol and its metabolites has been reported to involve glucuronidation exclusively [13]. This conclusion was based on the fact that hydrolysis with glucuronidase/sulfatase enzyme mixture was totally inhibited by D-glucaro-1,4-lactone, a specific glucuronidase inhibitor. Further support for the above conclusion was given by the equivalence of the hydrolysis when carried out with pure bovine β-glucuronidase.

Recently the sulfate conjugate of 4-hydroxypropranolol has been isolated from urine taken from humans and dogs dosed with propranolol [38, 39]. Further it has been shown that bisulfite, which is commonly used as an antioxidant in the enzyme hydrolysis inhibits the sulfatase component of the enzyme mixture used [38]. Therefore, in the presence of bisulfite the D-glucaro-1,4-lactone would completely inhibit the hydrolysis by the glucuronidase/sulfatase mixture and equivalent hydrolysis would be obtained with the pure glucuronidase.

To demonstrate the usefulness and reliability of our method the nature of the conjugation of propranolol and its metabolites was re-investigated using an experimental strategy similar to that previously reported [13]. Plasma samples were incubated at 37°C in the presence of ascorbic acid (0.2 mg/ml) with pure sulfatase (3 U Sigma S-1629, pH 7.0, 0.1 *M* Tris), pure bovine liver β-glucuronidase (1000 U Sigma G-0251, pH 4.8, 1.0 *M* sodium acetate), or the mixed enzymes as described earlier, with and without D-glucaro-1,4-lactone (10 mg, Calbiochem 557411). The results of these incubations are given in Table III. With ascorbic acid as antioxidant it is apparent that D-glucaro-1,4-lactone does not completely inhibit the hydrolysis of the conjugates of propranolol or its metabolites. This observed hydrolysis by the mixed enzyme in the presence of the glucuronidase inhibitor is assumed to be due to the presence of sulfate conjugates of propranolol and its metabolites. Support for this assumption is given by the comparable extent of hydrolysis observed with pure sulfatase and the apparent degree of sulfation given by the difference between the hydrolysis observed with the mixed enzymes (without inhibitor) and that observed with the pure glucuronidase.

If the interpretation of these results is correct then it is apparent that in dog plasma the conjugation of 4-hydroxypropranolol and N-desisopropyl-propranolol involves largely sulfation, whilst the conjugation of propranolol and propranolol glycol involves mainly glucuronidation (Table III). These conclusions will now await for experimental verification by the isolation and quantitation of both the glucuronide and sulfate conjugates of propranolol and the other metabolites as has been done with 4-hydroxypropranolol [39].

The method we have described is simple for the comprehensive analysis of propranolol and its metabolites featuring the efficient and simultaneous extraction of the analytes followed by their HPLC assay. The accuracy and reliability of our method has been well demonstrated and its usefulness in investigating the conjugation of propranolol and its metabolites shown. The minimum detectable levels (3× baseline) of the method using 1 ml of plasma are: 1.0

ng/ml propranolol, 6.0 ng/ml 4-hydroxypropranolol, 1.0 ng/ml N-desisopropylpropranolol and 2.5 ng/ml propranolol glycol.

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