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Fluorometric TLC Determination of Free and Conjugated Propranolol, Naphthoxylactic Acid, and *p*-Hydroxypropranolol in Human Plasma and Urine

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Abstract □ Sensitive, specific, and reproducible TLC methods are described for the determination of propranolol and its major metabolites in humans, conjugated propranolol, free and conjugated naphthoxylactic acid, and free and conjugated *p*-hydroxypropranolol. The drug or metabolites are extracted from plasma or urine with ether and applied to TLC plates of silica gel or microcrystalline cellulose. After development, the plates are scanned in a spectrodensitometer equipped to measure fluorescence in the UV and blue regions of the light spectrum. Quantitation is achieved by comparing the areas under the peaks obtained from the unknowns to those obtained from standards applied to the same plate. Limits of quantitation in plasma are: free propranolol, 2 ng/ml; free *p*-hydroxypropranolol, 10 ng/ml; conjugated propranolol, 15 ng/ml; total (free and conjugated) naphthoxylactic acid, 25 ng/ml; and conjugated *p*-hydroxypropranolol, 50 ng/ml. These methods were used to obtain plasma level data in a volunteer after one single dose of propranolol and in patients under propranolol therapy. The R_f values of some known metabolites of propranolol obtained in various TLC developing systems are also presented.

Keyphrases □ Propranolol—and major metabolites, fluorometric TLC analyses in human plasma and urine □ Fluorometric TLC—analyses, propranolol and major metabolites in human plasma and urine □ TLC fluorometry—analyses, propranolol and major metabolites in human plasma and urine □ Cardiac depressants—propranolol and major metabolites, fluorometric TLC analyses in human plasma and urine

In addition to β-adrenergic blockade, propranolol (I) has been reported to have antihypertensive, antianxiety, anticonvulsant, and antianginal effects (1–5). Over 95% of an orally administered dose of propranolol is metabolized to several different substances in humans (6–11). The major metabolites in humans were identified as conjugated propranolol (II), *p*-hydroxypropranolol (III), and naphthoxylactic acid (IV) in their free and conjugated forms.

GLC methods were reported for propranolol and *p*-hydroxypropranolol in biological fluids (12–14). For the routine analysis of unchanged propranolol, wet fluorometric procedures are commonly used (15–18). Recently, a high-pressure liquid chromatographic (HPLC) method was reported (19). The present report describes simple, sensitive, and highly specific methods to assay I, II, and free and conjugated III and IV in human plasma and urine.

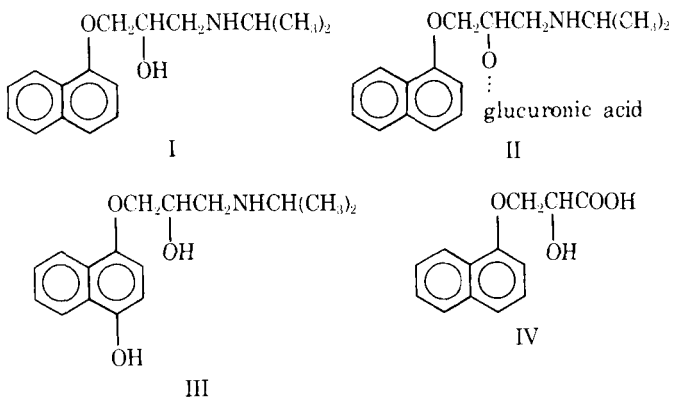
EXPERIMENTAL

Materials—All reagents and solvents were analytical reagent grade. TLC plates of silica gel or microcrystalline cellulose, 10 × 20 cm, were used. The plates were divided into 10 equal channels, 1 cm wide, with a scoring device[1]. Samples were applied to the plates with 25- or 50-μl microdispensers[2].

Acetate buffer, 0.1 *M*, pH 4.7, was prepared by dissolving 0.37 g of sodium acetate in 100 ml of distilled water and adjusting to pH 4.7 with acetic acid. Enzyme solutions[3] were prepared in distilled water. Standard solutions of I hydrochloride[4], III hydrochloride[4], and IV[4] were prepared in methanol. Ether[5], refluxed for 30 min in the presence of lithium aluminum hydride and distilled in an all-glass apparatus, was used for extracting III.

Determination of Free Propranolol—Human plasma or urine, 5 ml, was made basic with 1.0 ml of 1 *N* NaOH and shaken for 15 min with 40 ml of ether. After centrifugation, the organic phase was transferred and dried with 12 g of sodium sulfate. A 25-ml aliquot of the ether extract was transferred to a clean conical tube and evaporated under nitrogen[6] at 45°. The inside of the tube was washed with 1 ml of ethanol, and the solution was evaporated to dryness.

The dry residue was dissolved in 100 μl of ethanol, and aliquots of 25 μl were applied to a prescored TLC plate of silica gel 60[7]. Standard solutions of propranolol also were spotted on the same plate. The plate was



[1] SDA 320, Schoeffel Instrument Corp.
[2] Dialamatic, Drummond Scientific Co.
[3] Glusulase, Endo Laboratories.
[4] Imperial Chemical Industries, Great Britain.
[5] Mallinckrodt, Fisher, or Matheson, Coleman and Bell.
[6] N-Evap, Organomation Associates.
[7] E. Merck.

Table I—Recovery Experiments of Free Propranolol in Human Plasma and Urine

Plasma					Urine		
Amount Spiked, ng/ml	Amount Recovered, ng/ml				Amount Spiked, ng/ml	Amount Recovered, ng/ml	
	Run 1	Run 2	Run 3	Run 4		Run 1	Run 2
5	5	5	5	5	10	8	10
10	10	9	9	9	20	16	17
20	20	18	17	18	30	32	28
30	26	27	29	25	40	37	35
40	38	37	39	39	500	520	510
50	47	47	49	50	1000	940	890
	Mean	95.1%				Mean 92.4%	
	SD	6.5%				*SD* 8.7%	

developed in a saturated tank containing methanol (100 ml)–concentrated ammonium hydroxide (0.4 ml). After developing 12 cm, the plate was air dried and then sprayed with propylene glycol–water (50:50). It was then scanned in a spectrodensitometer[8] equipped with a density computer[9]. The light source was a xenon–mercury 200-w lamp[10].

The instrument was operated in the fluorescence mode with excitation set at 290 nm. Emission was filtered with a UV-transmitting, visible-absorbing filter[11] having a transmission band between 300 and 420 nm, with maximum transmission at 365 nm. A UV clear filter[12] also was used. Gain was set between 600 and 700 on the arbitrary scale of the spectrodensitometer. Scanning and chart speeds were 10.2 cm/min. Quantitation was achieved by comparing the areas under the peaks obtained from the unknowns to those obtained from the standards.

Determination of Conjugated Propranolol—Plasma or urine, 0.5 ml, was mixed with 2 ml of acetate buffer and 1 ml of an enzyme solution[3] containing 2000 units of β-glucuronidase and 500 units of sulfatase. The plasma mixture was incubated at 37° for 90 min, and the urine mixture was incubated at 37° for 17 hr. After incubation, 1.0 ml of 1 *N* NaOH was added, and the sample was shaken with 40 ml of ether for 15 min.

The ether phase was transferred and dried with 12 g of sodium sulfate. A 30-ml aliquot was transferred to a conical tube and evaporated under a nitrogen[6] stream at 45°. The inside of the tube was washed with 1 ml of ethanol, and this solution was evaporated to dryness. The dry residue was dissolved in 100 μl of ethanol, and aliquots of 25 μl of plasma extract or 10 μl of urine extract were applied to a prescored TLC plate of silica gel 60[7] along with standard solutions of propranolol. The plate was developed in a saturated tank containing 100 ml of benzene–isopropyl alcohol–dimethylformamide–acetic acid (70:10:10:10). The plate was sprayed and subjected to densitometry as described under *Determination of Free Propranolol.*

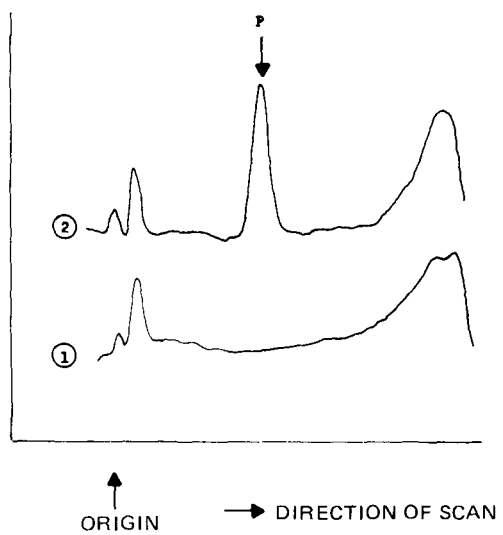


Figure 1—*Chromatograms of extracts of plasma obtained from a volunteer before (1) and 1 hr after (2) 40 mg po of propranolol hydrochloride. The* R_f *value of propranolol is indicated by the arrow.*

[8] Model SD 3000, Schoeffel Instrument Corp.
[9] Model SDC 300, Schoeffel Instrument Corp.
[10] Hanovia Lamp Division, Comrand Precision Ind.
[11] C.S. 7.51, Corning.
[12] C.S. 0.52, Corning.

The procedure outlined quantitates total propranolol. Conjugated propranolol is obtained by subtracting free from total drug.

Determination of Naphthoxylactic Acid—A 0.5-ml sample of plasma or urine was mixed with 2 ml of the acetate buffer and 1 ml of an enzyme solution[3] containing 2000 units of β-glucuronidase and 500 units of sulfatase. The mixture was incubated at 37° for the following time periods: plasma sample, 2 hr; and urine sample, 17 hr. After incubation, the sample was made alkaline with 1 ml of 1 *N* NaOH and extracted with 40 ml of ether. The ether extract was discarded, and the aqueous phase was acidified with 1.5 ml of 1 *N* HCl and extracted with 40 ml of ether for 15 min.

The ether extract was dried with 10 g of sodium sulfate, and a 25-ml aliquot was transferred to a clean conical tube and evaporated under nitrogen[6] at 45°. The inside of the tube was washed with 1 ml of ethanol, and the solution was evaporated to dryness. The dry residue was reconstituted with 100 μl of ethanol. Aliquots of 25 μl of the reconstituted plasma extracts and 5 μl of the urine extracts were applied to a prescored TLC plate of silica gel[13]. Standard solutions of IV were also applied to the same plate. The plate was developed in a saturated tank containing 100 ml of benzene–isopropyl alcohol–dimethylformamide–acetic acid (70:10:10:10). The plate was then sprayed and scanned in a spectrodensitometer as described under *Determination of Free Propranolol.*

This procedure measures total (conjugated and free) IV acid. Free acid can be determined by the same method, omitting the enzyme hydrolysis step.

Determination of *p*-Hydroxypropranolol—A 1.0-ml sample of plasma was mixed with 2 ml of acetate buffer and 0.5 ml of an enzyme solution[3] containing 10,000 units of β-glucuronidase and 2500 units of sulfatase. The mixture was incubated at 37° for 30 min. After incubation, 5 ml of pH 10 buffer[14] was added, and the sample was shaken with 40 ml of ether for 15 min.

For urine assay, a 0.1-ml sample was mixed with 0.5 ml of acetate buffer, 0.1 ml of 0.1 *N* ethylenediaminetetraacetic acid, and 0.1 ml of an enzyme solution containing 20,000 units of β-glucuronidase and 5000 units of sulfatase. The mixture was incubated at 37° for 20 min, after which 3 ml of pH 10 buffer[14] was added and the sample was extracted with 40 ml of ether.

The extract mixtures were centrifuged, and 30 ml of the ether phase was transferred to clean tubes and evaporated under nitrogen[6] at 45°. Remaining traces of water were removed by adding 4 ml of benzene–ethanol (5:2) to the tubes. The azeotropic mixture thus formed could be dried completely under nitrogen[6] at 45°. The dry residue was then dissolved in 100 μl of ethanol. The extraction and evaporation steps were carried out in 50-ml conical polypropylene tubes[15]. The reasons for using this type of tube are discussed later.

Aliquots of 25 μl of plasma extracts or 10 μl of urine extracts were applied to a TLC plate of microcrystalline cellulose[16]. Standard solutions of III were also applied to the same plate. The plate was developed in a saturated tank containing 100 ml of ethyl acetate–acetone–water (40:45:15). After development, the plate was air dried and scanned in a spectrodensitometer[8] operated in the fluorescence mode. Excitation wavelength was set at 300 nm, and emission was passed through a blue filter[17] with maximum transmission at 420–440 nm and a cutoff filter[18]. Quantitation was achieved by comparing the areas under the peaks obtained from the unknowns to those obtained from the standards.

[13] Macherey-Nagel. It is advisable to predevelop these plates to obtain a clean background.
[14] R-1280, Anachemia.
[15] Falcon 2070.
[16] Avicel, thickness 250 μm, Analtech.
[17] C.S. 5.60, Corning.
[18] C.S. 3.75, Corning.

Table II—Recovery Experiments of Propranolol in Human Plasma and Urine after Spiked Samples Were Submitted to Procedure to Assay Conjugated Propranolol

Plasma				Urine			
Amount Spiked, ng/ml	Amount Recovered, ng/ml			Amount Spiked, ng/ml	Amount Recovered, ng/ml		
	Run 1	Run 2	Run 3		Run 1	Run 2	Run 3
25	25	22	27	100	91	107	101
50	48	53	46	250	266	266	266
100	93	93	101	500	490	533	533
250	250	232	256	750	746	773	720
500	522	426	501				
	Mean 97.7%				Mean 101.9%		
	SD 6.3%				*SD* 5.2%		

This procedure measures total III. To assay free III, a 2-ml sample of plasma or urine was mixed with 4 ml of pH 10 buffer[14] and extraction was carried out as described. Conjugated III can be obtained by subtracting free from total metabolite.

Recovery Experiments—Samples of human plasma or urine were spiked with solutions of varying concentrations of I hydrochloride, IV, or III hydrochloride and subjected to the described analytical procedures.

Linearity of Response and Limit of Quantitation—Solutions of varying concentrations of I hydrochloride, IV, and III hydrochloride were spotted on TLC plates and subjected to chromatography to determine the linear ranges of instrument response. The limit of quantitation was set as an "area under the peak" of 10 units since the instrument could reliably integrate such areas under the conditions used.

***In Vivo* Experiment**—One healthy male volunteer was given 80 mg po of I hydrochloride[19]; blood samples were obtained at 0.5, 1, 2, 3, 4, 5, 6, 8, and 12 hr after dosing. A preadministration sample was also obtained. The blood was transferred to tubes containing ethylenediaminetetraacetic acid, mixed, and centrifuged. The plasma was transferred to clean tubes and frozen until assayed. Blood samples also were obtained from patients undergoing propranolol therapy and receiving the drug in 40-mg doses three or four times daily or in 160-mg doses four times daily. Blood specimens were treated as described.

RESULTS AND DISCUSSION

Free Propranolol—On a thin layer of silica gel, I has fluorescent properties similar to those observed in the wet state (15, 16). The emission filter[11] used transmits between 300 and 420 nm with maximum transmission at 365 nm. The clear filter[12] was used to filter off excitation radiation.

In the TLC system described, I had an R_f value of 0.34 and was separated from plasma and urine components. Figure 1 shows chromatograms obtained from the plasma of a human volunteer prior to and after administration of I hydrochloride. Propranolol was free of interference from its metabolites. Drug extraction was carried out at alkaline pH so that the acidic metabolites were not extracted. The basic metabolite, deisopropylpropranolol, and the neutral metabolite, 1-(α-naphthoxy)-2,3-propanediol, were separated from propranolol during chromatography, having R_f values of 0.15 and 0.84, respectively. Instrument response as determined by the area under the peak was linear over the range of 0–100 ng/spot, with a correlation coefficient of 0.999. The limit of quantitation was 2 ng/spot, equivalent to 2 ng/ml of plasma or urine.

Mean recovery from spiked plasma samples was 95.1 ± 6.5% *SD*, n = 24 (Table I). Mean recovery from spiked urine samples was 92.4 ± 8.7% *SD*, n = 12. All concentration units were calculated as nanograms of propranolol hydrochloride since it was the standard material used.

Spraying the TLC plates with propylene glycol–water is critical. Moisturizing the plate increases sensitivity 10-fold. Propylene glycol acts as a moisture-retaining agent and delays water evaporation from the plate. If a plate is sprayed properly, readings are stable for at least 10 min. This time is sufficient to scan a plate containing a minimum of six spotted channels. Since fluorescence intensity depends on moisture content, care must be taken to ensure even spraying of a plate.

The 10 × 20-cm plates are used because the width of the spray can be adjusted to cover the width of the plate completely. Uniform spraying is achieved by using an up and down motion. With the spray bottle 60 cm (24 in.) from the plate, 40 up and down passes are sufficient to provide adequate moisture, consuming about 30 ml of the spraying solution. Overspraying results in an erratic baseline, and underspraying causes a loss of sensitivity.

Conjugated Propranolol—The procedure described to assay this metabolite was also highly specific. In the TLC system used, the R_f values were: propranolol, 0.20; deisopropylpropranolol, 0.07; and 1-(α-naphthoxy)-2,3-propanediol, 0.67. There was no interference from the acidic metabolites due to the high extraction pH. Clean backgrounds were obtained from blank plasma and urine extracts.

Since the assay included an enzymatic hydrolysis step, it was essential

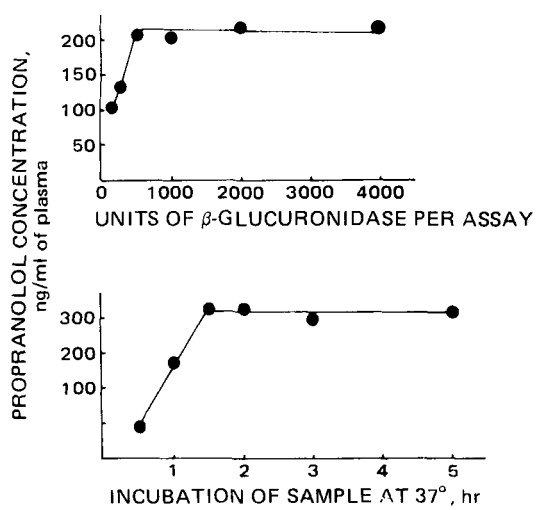


Figure 2—*Effects of enzyme concentration and time of incubation at 37° on the amount of propranolol released when a 0.5-ml plasma sample was subjected to hydrolysis in the presence of β-glucuronidase and sulfatase. Top: units of sulfatase were one-fourth those indicated for β-glucuronidase. Plasma sample was obtained from a patient receiving propranolol therapy (40 mg four times daily). Bottom: time of incubation of plasma sample at 37° in the presence of 2000 units of β-glucuronidase and 1000 units of sulfatase. Plasma sample was obtained from a patient receiving propranolol therapy (40 mg three times daily).*

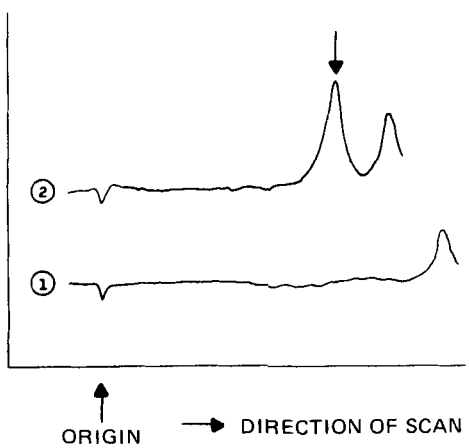


Figure 3—*Chromatograms of extracts of plasma obtained from a volunteer before (1) and 3 hr after (2) 40 mg po of propranolol hydrochloride. The R_f value of IV is indicated by the arrow.*

[19] Two 40-mg Inderal-40 tablets, Ayerst Laboratories.

Table III—Recovery Experiments of IV in Human Plasma and Urine

Plasma				Urine		
Amount Spiked, ng/ml	Amount Recovered, ng/ml			Amount Spiked, μg/ml	Amount Recovered, μg/ml	
	Run 1	Run 2	Run 3		Run 1	Run 2
250	254	231	246	2.5	2.6	2.5
500	546	473	440	5.0	4.7	5.3
750	763	695	675	7.5	6.4	6.6
1000	873	867	902	10.0	9.3	10.0
	Mean 94.4%				Mean 96.5%	
	SD 6.9%				*SD* 7.4%	

Table IV—Recovery Study of III in Human Plasma

Method for Free III				Method for Total III			
Amount Spiked, ng/ml	Amount Recovered, ng/ml			Amount Spiked, ng/ml	Amount Recovered, ng/ml		
	Run 1	Run 2	Run 3		Run 1	Run 2	Run 3
10	10	11	10	50	29	28	28
25	24	25	26	100	69	69	66
50	47	47	51	250	151	140	165
100	105	102	94	500	369	338	359
250	207	234	239	1000	688	646	688
500	390	440	480				
	Mean 96.4%				Mean 64.8%		
	SD 7.8%				*SD* 6.1%		

to determine if propranolol was stable under these conditions. Therefore, control plasma and urine samples were spiked with known amounts of propranolol and submitted to the procedure, including enzyme hydrolysis (Table II). Mean recovery of drug added to plasma was 97.7 ± 6.3% *SD*, n = 15. Mean recovery of drug added to urine was 101.9 ± 5.2% *SD*, n = 12. The limits of quantitation were 15 ng/ml of plasma and 50 ng/ml of urine.

Propranolol has been reported to be metabolized to a glucuronide conjugate in humans and animals (6, 9, 11, 20). The incubation periods and the enzyme concentrations used in enzyme hydrolysis were adequate to release the propranolol moiety maximally from the conjugate. Since no standard material of propranolol conjugate was available, plasma and urine samples were taken from patients receiving propranolol therapy and subjected to hydrolysis under varying conditions of enzyme concentrations and incubation times until a maximum amount of free propranolol was obtained (Fig. 2).

Compound IV—In the procedure described, IV was free of interference from propranolol and its known metabolites. The amine and neutral metabolites were removed during the alkaline extraction step. The acidic substances had the following R_f values: IV, 0.54; III, 0.06; *p*-hydroxy-1-(α-naphthoxy)-2,3-propanediol, 0.43; naphthoxyacetic acid, 0.71; α-naphthol, 0.74; and dihydroxynaphthalene, 0.61. Propranolol itself had an R_f value of 0.12. Compound IV was also free of interference from extractable plasma and urine components. Figure 3 shows a chromatogram of an extract of plasma containing the metabolite.

The areas under the peaks of IV were linear over the range of 0–250 ng/spot, with a correlation coefficient of 0.995. Mean recovery from spiked plasma was 94.4 ± 6.9% *SD*, n = 12 (Table III). Mean recovery from spiked urine was 96.5 ± 7.4% *SD*, n = 8. Since the spiked samples were submitted to enzyme hydrolysis before extraction, results of the recovery study indicated that IV was stable under the conditions used. The limits of quantitation were 25 ng/ml of plasma and 125 ng/ml of urine. The concentrations of enzymes and the incubation periods used were adequate to release the IV moiety maximally from the conjugates. Little is known about the conjugation of this metabolite. However, IV is present in plasma and urine in both free and conjugated forms. The question of the type of conjugate, glucuronide or sulfate, remains.

Compound III—This metabolite fluoresces strongly in the blue region of the light spectrum, with maximum emission between 400 and 450 nm.

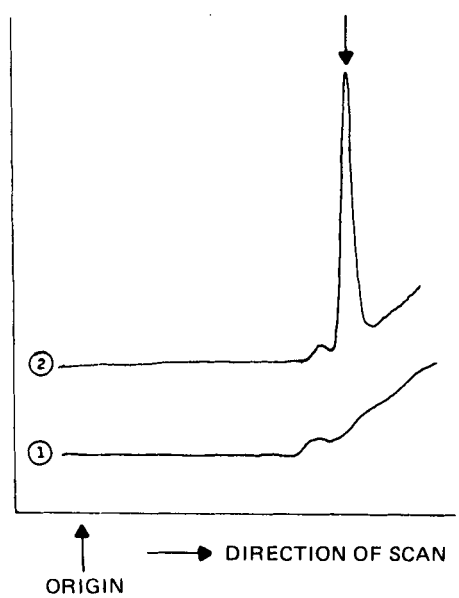


Figure 4—*Chromatograms of plasma extracts obtained from a volunteer before (1) and 0.5 hr after (2) 80 mg po of propranolol hydrochloride. The R_f value of III is indicated by the arrow.*

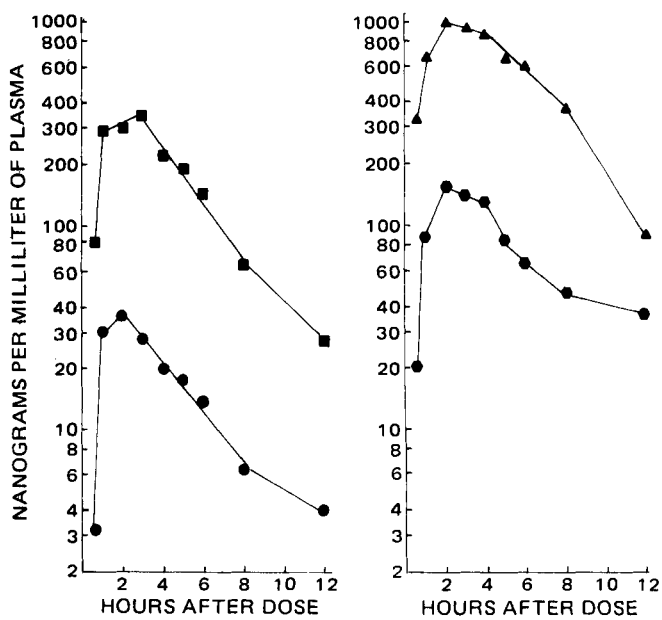


Figure 5—*Plasma levels of propranolol and metabolites following one single dose of 80 mg po of propranolol hydrochloride to a human volunteer. Key: ●, free propranolol; ■, conjugated propranolol; ●, total (free plus conjugated) III; and ▲, total (free plus conjugated) IV.*

Table V—Recovery Study of III in Human Urine

Method for Free III			Method for Total III		
Amount Spiked, ng/ml	Amount Recovered, ng/ml		Amount Spiked, mcg/ml	Amount Recovered, μg/ml	
	Run 1	Run 2		Run 1	Run 2
100	93	80	5.0	3.4	3.5
250	206	230	10.0	7.5	7.7
500	390	410	15.0	10.6	9.8
1000	790	830	25.0	20.3	20.3
	Mean 83.6%			Mean 73.5%	
	SD 5.7%			*SD* 5.9%	

Table VI—Plasma Levels (Nanograms per Milliliter) of Free Propranolol and Metabolites in Three Patients Receiving Propranolol Therapy

Patient	Dose, mg	Hours after Last Dose	Free Propranolol	Conjugated Propranolol	Total IV	Conjugated III
1	40 three times daily	4	25	348	684	377
2	40 four times daily	3	95	368	—[a]	478
3	160 four times daily	3	261	1630	1160	1233

[a] Not done.

In the system described, III had an R_f value of 0.57 and was separated from plasma and urine components. Chromatograms of plasma extracts are shown in Fig. 4. Compound III was also free of interference from the parent drug propranolol and its known metabolites. Compound IV and naphthoxyacetic acid were not extracted at pH 10.

Metabolites α-naphthol, dihydroxynaphthalene, 1-(α-naphthoxy)-2,3-propanediol, and its *p*-hydroxy derivative moved with the solvent front in the TLC system used (R_f 1.0). Propranolol and deisopropylpropranolol had R_f values of 0.59 and 0.53, respectively, and overlapped with the III peak. However, their maximum fluorescence emission (365 nm) was not transmitted through the blue filter[17] used and the compounds did not interfere with the assay. In fact, propranolol concentrations as high as 1.1 μg/ml of plasma and 13.3 μg/ml of urine did not interfere with the determination of III.

Data from recovery experiments done in plasma and urine are given in Tables IV and V, respectively. Mean recoveries of III from spiked plasma were 96.4% when the method for free III was used and 64.8% when the method for conjugated III was used. Similarly, mean recoveries from spiked urine were 83.6 and 73.5% when these respective methods were used. The lower recoveries obtained with the procedure used to assay the conjugate were due to the instability of III during enzyme hydrolysis. Compound III is itself metabolized to glucuronide and/or sulfate conjugates (9).

To determine if the III moiety was released completely from the conjugate during enzyme hydrolysis, plasma and urine samples from patients under propranolol therapy were incubated at 37° for different time periods and with different concentrations of enzyme solutions until maximum release of III was observed. Large concentrations of enzymes were used to keep the incubation time to a minimum since III degrades during incubation at 37°. Incubation of plasma or urine samples with the enzyme concentrations specified (see *Experimental*) was adequate to achieve maximum release after 30 min. Incubation for longer periods resulted in some loss of metabolite.

Limits of quantitation were: free III, 10 ng/ml of plasma or 100 ng/ml of urine; and conjugated III, 50 ng/ml of plasma or 2 μg/ml of urine.

Compound III is unstable and care must be exercised to prevent its degradation. Since it is sensitive to metal ions, blood specimens were always obtained in tubes containing ethylenediaminetetraacetic acid. It was extremely sensitive to peroxides present in the ether used for extraction. All traces of peroxides were removed by refluxing the solvent in the presence of lithium aluminum hydride followed by distillation in an all-glass apparatus immediately before use.

Compound III was heat labile and, therefore, enzyme hydrolysis could not be carried out at a temperature exceeding 37°. It was very labile when dried on a glass surface. When ether extracts of the metabolite were evaporated to dryness in glass tubes, complete loss of the metabolite was observed. Since this result could have been due to the presence of contaminating metal ions on the glass surface, evaporation was always carried out in polypropylene disposable tubes. Even with this type of material, however, it was essential that the tube be removed from the evaporation bath as soon as dried and that the residue be dissolved in ethanol immediately. Leaving the dry residue unattended even for a few minutes resulted in some loss of metabolite. Microcrystalline cellulose plates were used for TLC because silica gel, aluminum oxide, and polyamide plates caused degradation of the metabolite.

Compound III was not stable in refrigerated plasma. More than 80% of metabolite added to plasma samples was lost after 6 days of refrigeration at 4°. However, when kept frozen at −20°, the samples were stable for 1 month.

Levels of Propranolol and Metabolites in Human Plasma—To demonstrate the applicability of the methods, plasma level–time curves of propranolol and its three major metabolites obtained after administration of 80 mg po of propranolol hydrochloride to a human volunteer are shown in Fig. 5. In addition, plasma concentrations of the same substances in patients under propranolol therapy are given in Table VI. Free III could not be detected after a single dose of propranolol because its concentration was probably below the limit of detection of 10 ng/ml of plasma.

CONCLUSIONS

Sensitive, specific, and reproducible methods have been described for the determination of free propranolol and three of its metabolites in biological fluids. The methods are simple, relatively rapid, and especially useful when a large number of samples must be assayed. The methods offer several advantages over those already published.

The spectrofluorometric procedures (15–18), although convenient for analysis of a large number of samples, suffer from a lack of specificity since the metabolite deisopropylpropranolol is extractable under conditions specified in the assays. Similarly, the specificity of the HPLC method (19) with regard to the metabolites deisopropylpropanolol and 1-(α-naphthoxy)-2,3-propanediol has not been demonstrated. The GLC procedures (12–14) are specific and sensitive. However, in the authors' laboratory, they have proven time consuming and subject to complications because of a sensitive derivatization step.

The advantages of the TLC methods reside in their specificity and simplicity in so far as there is no need for derivatization, and the extraction, evaporation, and TLC spotting steps are quite straightforward. One person can easily analyze 20 samples in 1 working day.

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Determination of Indoprofen in Physiological Fluids by Reversed-Phase Liquid Chromatography

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Abstract □ A rapid, sensitive, reversed-phase high-performance liquid chromatographic procedure was developed for the quantitative analysis of indoprofen in plasma and urine. Minimal sample preparation is required for the analysis of unconjugated urinary or plasma drug levels. The method provided quantitative results for indoprofen levels of 0.5–50 μg/ml of plasma and 0.5–200 μg/ml of urine and had a lower detection limit of 1 ng. Total urinary indoprofen levels required enzymatic hydrolysis of the conjugated drug prior to analysis. Results are presented for the plasma and urinary excretion levels of indoprofen for a patient receiving a single oral dose.

Keyphrases □ Indoprofen—high-performance liquid chromatographic analysis in biological fluids □ High-performance liquid chromatography—analysis, indoprofen in biological fluids □ Analgesics—indoprofen, high-performance liquid chromatographic analysis in biological fluids

Indoprofen, *dl*-2-[4-(1-oxo-2-isoindolinyl)phenyl]propanoic acid, has analgesic activity in animals and humans (1–4). In humans, indoprofen appears unchanged in plasma (5) and enantiomeric enrichment is not significant (6). Tosolini *et al.* (7) reported that indoprofen was excreted as the glucuronide conjugate and that enzymatic hydrolysis was required prior to determining urinary excretion levels.

GLC methods for the analysis of indoprofen in plasma and urine have been reported (6–8). While each method has analytical validity and sufficient sensitivity, they all require extraction of indoprofen from the biological fluid and derivatization to provide the necessary volatility for GLC analysis. These extraction and derivatization steps are time consuming and, consequently, expensive for extensive bioavailability studies.

To overcome these difficulties in sample preparation and to maintain the necessary selectivity and sensitivity for low level analysis, a method was developed using reversed-phase high-performance liquid chromatography (HPLC). The method is applicable to both urine and plasma samples containing indoprofen and has sensitivity at the nanogram level.

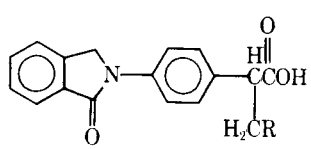
EXPERIMENTAL

Reagents—Acetonitrile[1] was used as received. The water was first deionized and then distilled to remove any contaminants. For enzymatic hydrolysis of conjugated drug, aryl sulfatase[2] containing β-glucuronidase was used.

Reference Compound and Internal Standard—A reference standard of indoprofen[3] (I) was used to develop the analytical method. The internal standard (II) selected for quantitation was the pentanoic acid homolog of indoprofen[3].

HPLC Conditions—To determine the optimal wavelength for UV detection of indoprofen, reference standard was dissolved in methanol and its UV spectrum was obtained. The standard has a λ_{max} at 282 nm with an absorptivity (ε 282) of 14,200 ($E^{1\%}_{1\,cm}$ = 505). The reported $E^{1\%}_{1\,cm}$ value for indoprofen is 500 ± 25 (λ_{max} 283)[4]. A 280-nm filter was used.

The liquid chromatograph was constructed from components and consisted of a high pressure pump[5], a loop injector[6], and a UV detector[7]. The initial HPLC conditions used for urinary analysis consisted of a Zorbox ODS[8] (250 × 2 mm i.d.) column and an eluent of 40% acetonitrile



I: R = H
II: R = CH_2CH_3

[1] Nanograde, Mallinckrodt Chemical Works, St. Louis, Mo.
[2] Sigma Chemical Co., St. Louis, Mo. (31,000 units/g of solid; 1 unit will hydrolyze 1.0 μmole of nitrocatechol sulfate/hr at pH 5.0 at 37°).
[3] Adria Laboratories, Columbus, Ohio.
[4] Dr. Werner Hausmann, Adria Laboratories, Columbus, Ohio, personal communication.
[5] Model 6000A, Waters Associates, Milford, Mass.
[6] Model UK6, Waters Associates, Milford, Mass.
[7] Model 440, Waters Associates, Milford, Mass.
[8] DuPont Co., Wilmington, Del.