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Short Communication

Simplified high-performance liquid chromatographic method for propranolol and five metabolites in liver perfusate, rat serum and dog plasma

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Abstract

A new method has been developed for the analysis of propranolol plus five major metabolites in perfusate from isolated liver preparations, and also in rat serum and dog plasma. The regional isomers of hydroxypropranolol are clearly separated within a total run time of less than 15 min. The basic and neutral metabolites are extracted and analysed together, while the acidic metabolites are extracted in a second step. The new assay is more simple and time efficient than previously published methods.

1. Introduction

Propranolol (PL) and some other lipophilic, high-first-pass drugs, *e.g.* metoprolol, labetolol and propafenone, show increased bioavailability when given with food, even though they are completely absorbed by fasting subjects [1]. The interaction between food and these drugs has been shown to be located in the liver but the mechanism has not been completely elucidated despite intense investigation over the last fifteen years. A transient increase in blood flow associated with food ingestion has been shown to play a possible role [2], but the mechanism is likely to be complex, involving metabolism and hepatic uptake of drug as well. Because of the difficulty to study this problem in humans, rat and dog have been used as animal models for the study of the various mechanisms that have been proposed to explain this interaction [3–6]. Our aim was to study the mechanisms that might contribute to the food–propranolol interaction in the isolated, perfused rat liver and in a chronically instrumented dog model. A method for the quantitation of propranolol and its metabolites in these model systems is therefore required.

The major routes of the propranolol metabolism are shown in Fig. 1. A number of methods have been published for the determination of propranolol and one or two metabolites, see ref. 7, but only a few cover five or more metabolites [7–9]. The method described here was initially developed to simplify one of the previously described methods [7], to decrease the time needed for analysis and to increase its selectivity. To achieve this goal the number of extraction steps was decreased and the length of column was increased. The new method is useful for the

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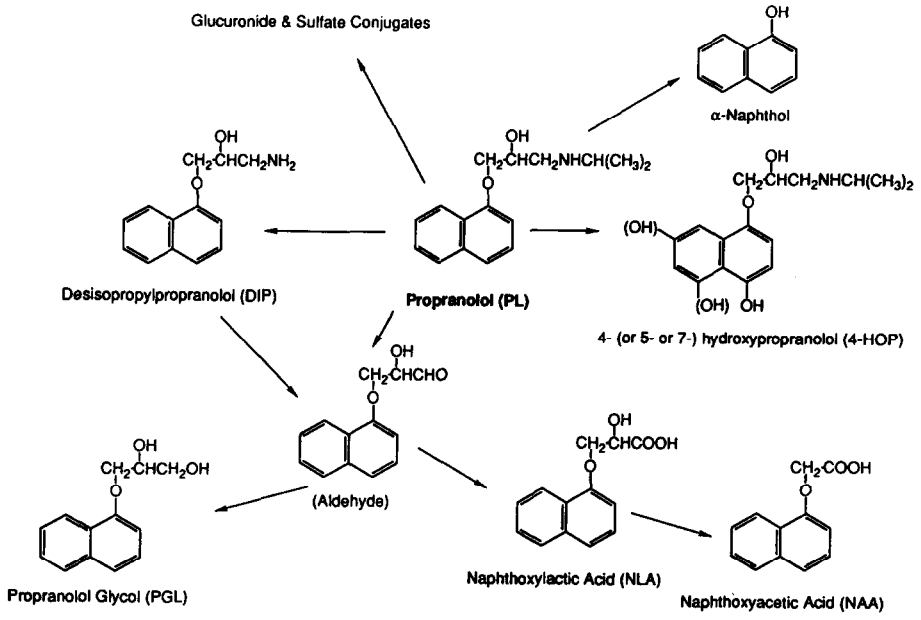

Fig. 1. The main pathways of propranolol metabolism.

efficient measurement of propranolol and up to eight of its metabolites, 4-, 5- and 7-hydroxypropranolol (4-, 5- and 7-HOP), N-desisopropylpropranolol (DIP), propranolol glycol (PGL), naphthoxylactic acid (NLA), naphthoxyacetic acid (NAA) and α-naphthol in the effluent buffer from our single-pass isolated, perfused rat liver system. The method has also been adapted for rat serum and dog plasma.

2. Experimental

2.1. Chemicals

PL hydrochloride was obtained from Sigma (St. Louis, MO, USA) and the metabolites, 4-HOP, DIP, PGL, NLA, 1-NAA and 4-methylpropranolol (4-MEP, internal standard) were gifts from I.C.I. (Macclesfield, UK). 2-NAA (internal standard) was from Aldrich (Milwaukee, WI, USA). Two regional isomers of HOP, 5-HOP and 7-HOP, were kindly supplied by Dr. W. Nelson, University of Washington. β-Glucuronidase was Type H3 crude extract from *Helix pomatia* (Sigma). All solvents were HPLC grade (BDH, Edmonton, Alb., Canada). Other chemicals used were of the highest grade available.

2.2. Preparation of standards

Stock solutions of PL hydrochloride (450 μg/ml), 4-HOP hydrochloride (45 μg/ml), DIP hydrochloride (20 μg/ml), PGL (4.5 μg/ml), NLA (6.5 μg/ml) and NAA (4.5 μg/ml) were prepared in 10% methanol (v/v) in water and stored at −20°C, except for 4-HOP to which 5% (w/v) ascorbic acid was added as an antioxidant. Shortly before use, a working solution was prepared by diluting the stock solution × 25 with Krebs bicarbonate buffer [10]. To a set of seven culture tubes containing 100 μl of 10% (w/v) ascorbic acid were added 0, 20, 50, 100, 250, 500 and 900 μl of the working solution. The volume of each sample was adjusted to 1.1 ml with Krebs buffer to complete the preparation of the standard solutions.

Two internal standards were used, 4-MEP for the basic and neutral metabolites, and 2-NAA for the acidic metabolites of propranolol. Working solutions of 1.5 μg/ml of 4-MEP and 2 μg/ml of 2-NAA were prepared. To all stan-

dard, blank and unknown samples 100 μl of each of the internal standard solutions was added.

2.3. Sample preparation

Rat livers were perfused via the hepatic portal vein with oxygenated Krebs bicarbonate buffer, pH 7.4, containing 2 g/l of glucose and varying amounts of propranolol, depending on the purpose of the experiment. Samples (1 ml) of liver effluent were collected from the vena cava into 16 × 100 mm culture tubes and closed with PTFE-lined caps. The tubes contained 100 μl of 10% ascorbic acid as an antioxidant to protect 4-HOP. The samples were stored at −20°C overnight.

2.4. Extraction procedures

The basic and neutral metabolites were extracted by adding 2 ml of 1 *M* sodium carbonate (pH 10) and 5 ml of diethyl ether to each 1-ml sample. The samples were vortex-mixed for 3 min, then centrifuged at 1200 *g* for 10 min to separate the phases. The upper organic phase was transferred into empty culture tubes and evaporated under a stream of nitrogen at room temperature. The samples were reconstituted in 2 ml of mobile phase. The lower aqueous phase was acidified to pH 3.2 (same as the mobile phase) with 250 μl of 6 *M* HCl and injected directly onto the HPLC system to quantify the acidic metabolites.

2.5. Sample preparation and extraction procedure modifications for use with rat serum and dog plasma

The extraction of PL and its basic and neutral metabolites remained the same as above. To extract the acidic metabolites, the aqueous phase and the small amount of organic phase remaining from the previous extraction step was first acidified with 2 ml of 6 *M* HCl, and then 5 ml of ether was added. The samples were vortex-mixed and centrifuged for 10 min at 1200 *g* and the organic layer was removed, dried under a stream of nitrogen, and reconstituted in 2 ml of mobile phase.

2.6. Chromatographic conditions

The parent drug and metabolites were separated by HPLC on a 250 × 4 mm I.D., 5-μm particle size LiChroSpher RP-18 column (E. Merck, Darmstadt, Germany) using a mobile phase of water–acetonitrile–methanol (45:22:33, v/v), containing 0.033% (v/v) triethylamine and 0.044% (v/v) of concentrated phosphoric acid, pH 3.2, pumped at a flow-rate of 1.0 ml/min. The instrumentation included a Waters 510 pump, Waters 710B WISP Autoinjector (Millipore-Waters, Missisauga, Ont., Canada), Perkin Elmer Model LS-4 fluorescence detector (Perkin-Elmer, Winnipeg, Man., Canada), and a Waters Baseline data system. Two detector sensitivities were required for the quantitation of both propranolol and its basic and neutral metabolites in the same run since propranolol was present in much greater quantities than the metabolites. The detector was programmed to adjust its emission wavelength according to Table 1. For analysis of the acidic metabolites, the excitation wavelength was 300 nm and the emission wavelength was 375 nm for the entire run. The sample injection volumes were 100 μl for the basic and neutral fraction and 250 μl for the acidic fraction.

2.7. Calibration

Calibration of the chromatograms was accomplished using the internal standard method. Linear calibration curves were produced from the peak-height ratios of PL and its metabolites to the appropriate internal standard *vs.* the concentration in the standard samples.

Table 1
Time program for fluorescence detector

Time (min)	Excitation λ (nm)	Emission λ (nm)
0	300	375
8.0	300	440
11.5	300	375

2.8. Treatment with β-glucuronidase

The 1-ml samples of liver perfusate were incubated with 700 units of β-glucuronidase at 37°C for 4 h and analyzed for PL, 4-HOP, and PGL. To quantitate the conjugated species, the concentrations of PL and metabolites measured without β-glucuronidase incubation were subtracted from the concentrations measured after hydrolysis. Conjugated DIP, NLA or NAA were not detected in liver perfusate.

3. Results and discussion

Each of the previously described assay methods for PL and several of its metabolites has advantages and drawbacks. The first of these methods was developed to measure PL and six metabolites in urine but not in serum or plasma [9]. The assay described by Kwong and Shen [8] is quite comprehensive and conditions were described for analysis of PL and its metabolites, 4-, 5- and 7-HOP, DIP, PGL and NLA plus conjugates of PL and the HOP isomers in serum, bile and urine. For serum samples, a two-step extraction was used. The basic and neutral metabolites were extracted together, followed by a second step to extract the acidic metabolites. The HPLC run time, however, was 40 min, even when the column was heated. Qureshi and Buttar [7] described a procedure with a shorter run time (10–15 min) but their extraction procedure involved separation of the neutral metabolites from the basic metabolites. This added an extraction step and increased the number of injections per sample to three instead of two, as each fraction was analyzed separately. It is also unclear whether their method can separate 4-HOP from the regional isomers 5- and 7-HOP. This is important because the rat produces measurable quantities of all three isomers.

With the newly developed method, one extraction step separated PL and its basic and neutral metabolites from the acidic metabolites. Because the effluent buffer was already relatively clean and protein-free compared with serum or plasma, the acidic fraction could be injected directly onto the column after pH adjustment. This represents a significant decrease in processing-time for perfusate samples. For serum or plasma, the presence of protein precluded direct injection, and thus a second extraction step was necessary. Even so, only two injections per sample were necessary under the described HPLC conditions. Fig. 2 shows the flow charts of the extraction procedures.

The efficiency of the method was retained in the presence of high (>15 μg/ml) concentrations of propranolol, while retaining appropriate sensitivity for the metabolites, some at concentrations <50 ng/ml. By programming the detector to temporarily change the emission wavelength from 375 to 440 nm, the sensitivity around the retention time of propranolol was reduced.

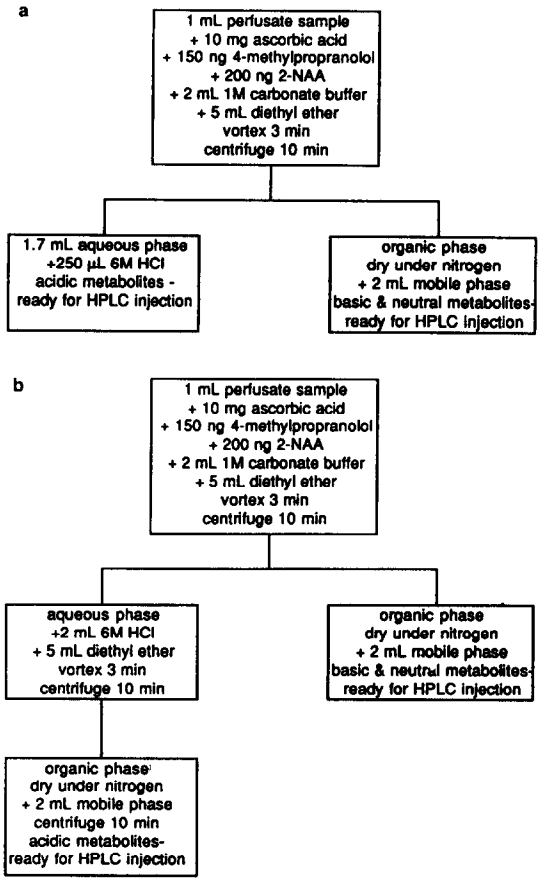

Fig. 2. Flow charts of the extraction procedures from (a) liver perfusate, and (b) rat serum or dog plasma.

This procedure allowed the accurate quantitation of all important peaks in the basic extract in one run.

The run times were *ca.* 15 min, even though a long (25 cm *vs.* 15 cm [7]) column was used. Within this time frame, this column was able to clearly separate PL, the regional isomers of HOP plus DIP, PLG, NLA, NAA and α-naphthol. Some unidentified PL-related peaks were present in our samples, and some of these may relate to metabolites separated by Kwong and Shen but not identified or quantitated in their serum extracts, *e.g.* 2-HOP and 4-OH-NLA [8]. Fig. 3 shows chromatograms of a blank perfusion effluent sample, a sample spiked with PL and metabolites, and a sample of liver effluent 70 min after the start of a 20 μg/ml infusion of PL.

Although two regional isomers of HOP, 5-HOP and 7-HOP, were separated and detected they were not quantitated as we did not have a sufficient quantity of standard. The retention times of the three isomers were, in minutes: 5-HOP, 3.98; 4-HOP, 4.42; 7-HOP, 5.36. 5-HOP produced peaks of approximately the same size as 4-HOP, but was apparently not conjugated in the isolated liver system, while 7-HOP produced higher peaks than 4-HOP, both before and after β-glucuronidase hydrolysis (See Fig. 3 for a pre-hydrolysis sample). α-Naphthol (retention time 10.38 min) was also separated but was not quantitated because it appeared in very low concentrations in the liver perfusate. Thus, in total eight metabolites that matched the retention times of known standard compounds were separated.

Data from nine runs performed on separate days were used to assess the assay in Krebs buffer/rat liver perfusate. The calibration curves were linear over the appropriate ranges with correlation coefficients near unity (Table 2). The slopes and intercepts derived by linear regression and the ranges of concentrations measured are also shown in Table 2. The intercepts were minimal and the slopes were reproducible between days. The extraction efficiencies were high for all the compounds measured, except for DIP (Table 3). The extraction efficiencies for all of the measured metabolites were similar to those

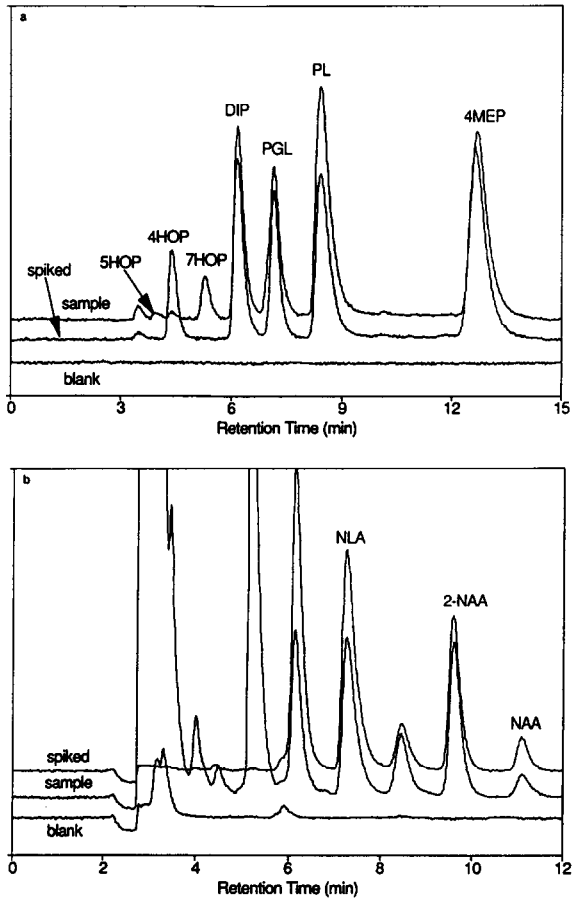

Fig. 3. Overlaid chromatograms of (a) PL and the basic and neutral metabolites, and (b) the acidic metabolites, from a blank sample, a sample spiked with PL and its metabolites, and a sample of liver effluent taken 70 min after the start of a 20 μg/ml infusion of PL. The retention times and the concentrations in the spiked sample were: 4HOP, 4.42 min, 1500 ng/ml; DIP, 6.26 min, 600 ng/ml; PGL, 7.34 min, 100 ng/ml; PL, 8.58 min, 9000 ng/ml; 4-MEP (internal standard for basic and neutral metabolites), 12.98 min, 1500 ng/ml; NLA, 7.18 min, 220 ng/ml; 2-NAA (internal standard for acidic metabolites), 9.43 min, 2000 ng/ml; 1-NAA, 11.28 min, 80 ng/ml.

reported by other investigators [7]. The limit of detection reported in Table 2 was arbitrarily set based on the range of steady-state metabolite concentrations observed in the rat liver perfusates. The sensitivity can be (and has been for studies in dogs) extended several-fold if necessary, for instance, by increasing the sample size and/or by decreasing the reconstituted volume

Table 2
Calibration curve performance parameters

Metabolite	Range (ng/ml)	Detection limit (ng/ml)	Slopes		Intercepts (mg/ml)		Correlation coefficient (mean) (r)
			Mean	S.E.	Mean	S.E.	
4-HOP	180–1800	95	1690	33	11	2.9	0.9993
DIP	60–1350	31	878	32	3.6	2.0	0.9994
PGL	14–315	8	242	4	−0.2	1.0	0.9998
PL	600–13500	533	9148	118	−35	15	1.0000
NLA	18–405	8	170	3	1.3	0.3	0.9997
NAA	24–135	20	399	1	−0.5	0.3	0.9996

Data are given as mean ± standard errors (S.E., $n = 9$).

Table 3
Recovery of drug and metabolites from extracts

Metabolite	Mean extraction efficiency ($n = 3$)
4-HOP	84
DIP	45
PGL	102
PL	92
NLA	101
NAA	98

of the extracted sample and reducing the amount of internal standard added.

Acidification of the aqueous phase with 6 *M* HCl after extraction of the basic metabolites from rat serum or dog plasma resulted in the formation of precipitates. After the metabolites were extracted into ether, some solids appeared when the solvent was evaporated and the sample reconstituted in mobile phase. For this reason, the samples were centrifuged. No column problems have been encountered when the supernatant was injected directly. Fig. 4 shows chromatograms from blank and spiked rat serum and Fig. 5 is from blank and spiked dog plasma.

Because we were working with a single-pass system, less Phase II metabolism may have occurred compared with a recirculating or an *in*

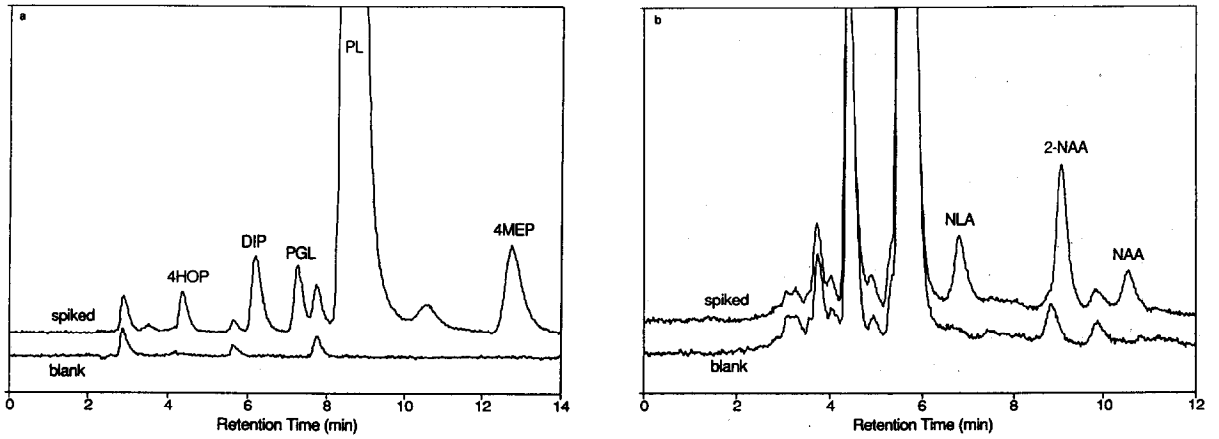

Fig. 4. Overlaid chromatograms of (a) PL and the basic and neutral metabolites, and (b) the acidic metabolites, from a blank sample and a sample spiked with PL and its metabolites in rat serum. The retention times and the concentrations in the spiked sample were: 4HOP, 4.42 min, 650 ng/ml; DIP, 6.26 min, 250 ng/ml; PGL, 7.34 min, 45 ng/ml; PL, 8.58 min, 4000 ng/ml; 4-MEP (internal standard for basic and neutral metabolites), 12.98 min, 700 ng/ml; NLA, 7.18 min, 90 ng/ml; 2-NAA (internal standard for acidic metabolites), 9.43 min, 2000 ng/ml; 1-NAA, 11.28 min, 90 ng/ml.

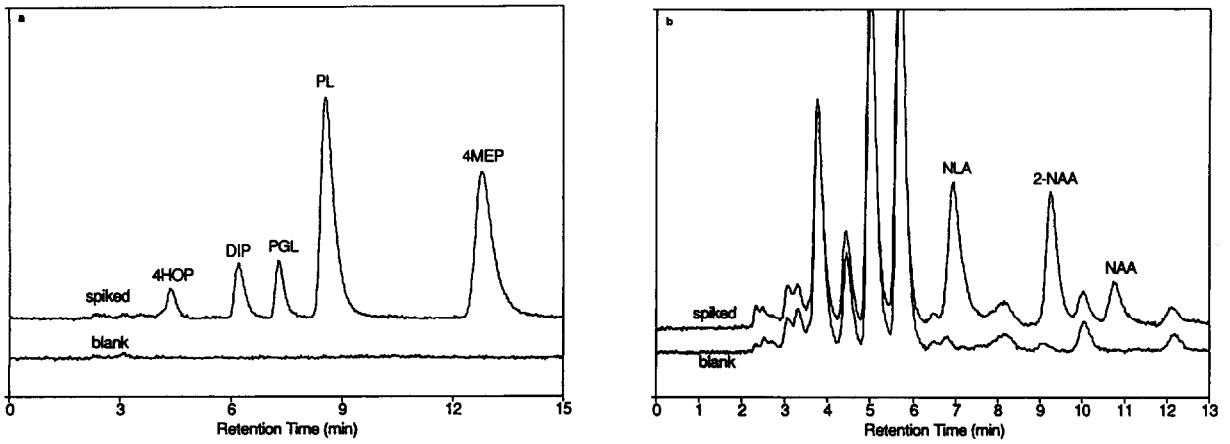

Fig. 5. Overlaid chromatograms of (a) PL and the basic and neutral metabolites, and (b) the acidic metabolites, from a blank sample and a sample spiked with PL and its metabolites in dog plasma. The retention times and the concentrations in the spiked sample were: 4HOP, 4.42 min, 450 ng/ml; DIP, 6.26 min, 180 ng/ml; PGL, 7.34 min, 40 ng/ml; PL, 8.58 min, 11000 ng/ml; 4-MEP (internal standard for basic and neutral metabolites), 12.98 min, 1100 ng/ml; NLA, 7.18 min, 190 ng/ml; 2-NAA (internal standard for acidic metabolites), 9.43 min, 1700 ng/ml; 1-NAA, 11.28 min, 100 ng/ml.

vivo system. This may be the reason that much less β-glucuronidase was required to hydrolyse the conjugated PL and metabolites in liver perfusate compared to the amount used by Kwong and Shen for rat serum [8]. This was important because at higher concentrations, a glucuronidase-related peak interfered with 5-HOP and 4-HOP and DIP. At the concentration of β-glucuronidase used, the baseline was almost undisturbed. We confirmed the amount of β-glucuronidase required by performing incubations at different times and dilutions. A ten-fold excess of enzyme, which was ten times less than that used by Kwong and Shen, was used in sample incubations [8].

In conclusion, the HPLC method described here for PL and its major metabolites is comprehensive, and offers the advantages of simplicity and a short time needed for assay. These qualities make it useful for pharmacokinetic studies of propranolol metabolism.

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5. References

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