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A fluorimetric liquid chromatographic method for the determination of propranolol in human serum/plasma

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Abstract

A simple, rapid, and sensitive fluorimetric-high-performance liquid chromatographic method for the determination of propranolol in human serum/plasma has been developed, without the need for solvent extraction. The procedure required 200 µl of serum/plasma, and the addition of 1 ml of acetonitrile for protein precipitation followed by vortexing and centrifugation at 10 000 *g*. The clear supernatant was evaporated to dryness under a stream of nitrogen at 50–60°C, the residue was reconstituted in 100 µl of methanol, and a 90 µl portion was injected onto the high-performance liquid chromatograph for propranolol quantitation. Chromatography was accomplished using a Hypersil® cyano column, a mobile phase of acetonitrile–aqueous acetic acid (1%) containing 0.2% triethylamine (35:65, v/v) (pH 3.6), a flow rate of 1.5 ml min^{-1}, a fluorescence detector set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm, and using pronethalol as the internal standard. Retention times for pronethalol and propranolol were 7.5 min and 9.5 min, respectively. Standard curves were linear in the range 5–200 ng ml^{-1}. Relative standard deviations for both inter-day and intra-day precision analysis were less than 7% for serum. No interference was observed from endogenous serum/plasma components. Specificity was shown for some, but not all, commonly coadministered drugs tested. The advantages of this method include good precision, low sample volume, good reproducibility and recovery, and high sensitivity.

Keywords: Beta blocker; Fluorescence detection; HPLC; Plasma; Pharmacokinetic analysis; Propranolol; Reversed-phase chromatography; Serum

1. Introduction

Since its discovery in 1964, propranolol, a β-adrenergic blocking agent has been widely used in the treatment of angina, hypertension, cardiac arrhythmias, migraine headaches and thyrotoxicosis. Its safety and efficacy has been well documented in over 2500 published clinical studies and is one of the most widely prescribed β-blockers licensed for use in the United States [1,2]. Propranolol is eliminated almost exclusively by hepatic metabolism and more than 18 metabolites have been identified, with at least four of these having pharmacological activity [3]. Serum/plasma levels encountered during therapy with propranolol may range from a low of 1 to above 100 ng ml^{-1}.

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An extensive overview of the different bioanalytical methods for propranolol, their advantages and disadvantages have been discussed by Sood et al. [4]. Most of the reported methods require extensive sample work-up and laborious time-consuming, extraction procedures. Although many of these methods offer the advantage of being specific, they do require large volumes (1–3 ml) of serum/plasma and/or are not economically feasible for routine use in most bioavailability studies or for their use in pharmacokinetic studies where small volumes of serum/plasma may only be obtained. The use of protein-coated ODS columns for the direct analysis of six drugs (including propranolol), omitting the deproteinization of the plasma, showed excellent accuracy and reproducibility, but the sensitivity of the assay was not reported [5]. Arunyanart and Cline Love [6] reported the use of micellar liquid chromatography using sodium dodecyl sulfate with 10% propanol as the mobile phase; however, this method was not applicable to the analysis of low levels of the drugs investigated.

This paper describes a simple, rapid and sensitive fluorimetric HPLC method for the determination of propranolol in human serum/plasma without solvent extraction. Experimental conditions, linearity, sensitivity, recovery, column-to-column reproducibility and drug interference studies are discussed.

2. Methods

2.1. Chemicals and reagents

HPLC grade methanol and acetonitrile, and A.C.S. grade glacial acetic acid and toluene were obtained from Fisher Scientific Co., Fair Lawn, N.J. Triethylamine (TEA), gold label, and dimethyldichlorosilane (99%) were purchased from Aldrich Chemical Co., Milwaukee, WI. Propranolol hydrochloride was manufactured by Lusochima S.p.A. Italy, and pronethalol hydrochloride and 4-hydroxypropranolol were provided by Imperial Chemical Industries, UK.

2.2. Glassware

All glassware was thoroughly cleaned and silanized with 5% dimethyldichlorosilane in toluene (w/v), rinsed with toluene followed by methanol, and allowed to dry in air prior to use.

2.3. Instrumentation

Reversed-phase high-performance liquid chromatography (HPLC) was performed using an M6000 A solvent delivery system, a WISP 710B automatic injector (Waters Associates, Inc., Milford, MA), and a Hypersil® CN column (250 × 4.6 mm^2; 5 μm) fitted with a cyano guard column (10 × 4.6 mm^2) (Alltech Associates, Deerfield, IL). The column eluant was monitored with a fluorescence spectrophotometer model 204-A (Perkin-Elmer, Norwalk, CT) equipped with a microflow cell accessory kit. The excitation wavelength was 230 nm, and the emission wavelength was 340 nm, with both slit openings set at 10 nm. The fluorimeter was operated at a sensitivity range of 1 and a power gain of 3. The output was recorded on a 3390A integrator (Hewlett Packard, Avondale, PA).

2.4. Mobile phase

The mobile phase was 35:65 (v/v) acetonitrile–acetic acid containing TEA solution (36:65, v/v) (0.2 ml of triethylamine in 1% acetic acid solution in water (pH 3.6)). The mobile phase was mixed thoroughly and deaerated using a vacuum pump at a flow rate of 1.5 ml min^{-1}.

2.5. Standard curve

Daily standard curves were prepared as follows. A 1 μg ml^{-1} solution of propranolol (as the free base) in water was prepared from a propranolol hydrochloride stock solution in water (1.14 mg ml^{-1}). To a 0.5 ml aliquot of this standard solution, 2 ml of drug free serum/plasma were added to make up a 200 ng ml^{-1} standard. This was then serially diluted with serum/plasma to yield concentrations of 5, 10, 25, 50, and 100 ng ml^{-1}

2.6. Analytical procedure

To 200 μl of either serum from a subject or spiked serum/plasma in a 15 ml Corex tube were added 1 ml of acetonitrile and 25 μl of pronethalol hydrochloride (2 μg ml^{-1}). The mixture was vortexed for 15 s and then centrifuged for 10 min at 10 000 rev min^{-1}. The clear supernatant was transferred to a disposable tube and evaporated to dryness under a stream of nitrogen in a water bath maintained at 50–60°C. The residue was reconstituted in

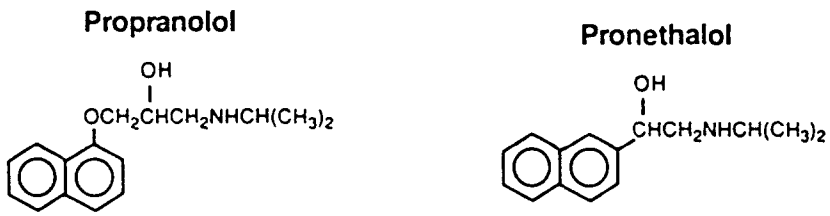


Fig. 1. Structural formulas of propranolol and pronethalol.

100 µl methanol, vortexed briefly, and 90 µl were injected onto the column. The peak area ratio of propranolol to the internal standard was determined. The regression line of peak area ratios versus standard concentrations was calculated, and the concentration in the unknown sample was estimated from the regression line.

2.7. Recovery

The recovery of propranolol from human serum/plasma was performed in the concentration range 2-200 ng ml^{-1}. Propranolol standards in methanol were prepared in an identical concentration range. The percent recovery was calculated from the recovered peak area ratio of the drug in the serum/plasma to the methanolic standard multiplied by 0.5 multiplied by 100. The 0.5 factor corrects for the dilution factor.

2.8. Precision

The intraday (within-day) precision was determined using three replicate analyses of spiked serum samples at five different concentrations: 5, 10, 25, 50 and 100 ng ml^{-1}. For the determination of the inter-day (between-day) precision, spiked serum controls were prepared in the concentration range 5-100 ng ml^{-1}. Controls at different concentrations stored at −20°C were analyzed with each standard curve over a period of a month.

2.9. Specificity

Heparin-coagulated blank plasma and serum obtained from different subjects ($n = 10$) (Brigham and Women's Hospital, Boston, MA) was analyzed using the described procedure. The possible interferences from normal plasma constituents were examined by inspecting the chromatograms.

2.10. Drug interference studies

Propranolol is often coadministerd with other cardiac drugs such as antiarrhythmic, antianginal agents and other antihypertensive agents. A number of drugs were tested for their potential interference in the chromatographic elution by injecting aliquots of stock solutions of these compounds.

3. Discussion

A number of HPLC methods reported in the literature [1,3-9] were evaluated for the analysis of propranolol in serum/plasma, but none provided satisfactory results in terms of sensitivity and reproducibility. This necessitated the development of an improved, sensitive plasma analytical HPLC method for propranolol which would be simple and also would demonstrate good column-to-column reproducibility.

To obtain a good separation between propranolol and pronethalol, different types of columns and mobile phases were examined. Pronethalol hydrochloride, due to its structural similarity (Fig. 1) to propanolol hydrochloride, was chosen as the internal standard to normalize erratic recoveries and to improve the precision of the analysis. The selection of the cyano packing material was based on its hydrophilicity, better peak shape for propranolol, and column preformance as measured by shorter retention time, efficiency, selectivity and resolution.

Both propranolol and 4-hydroxypropranolol exhibit natural fluroescence, allowing their detection with use of a fluorescence detector. The excitation wavelengths (230 or 295 nm) are the same for both compounds; however, the fluroescence emission wavelengths for propranolol and 4-hydroxypropranolol in aqueous solution are 340 nm and 435 nm, respectively [10]. The present method uses a particular setting of excitation and emission wavelengths (230 nm

and 340 nm, respectively) to measure propranolol. Furthermore, a compromise emission wavelength setting between 340 and 435 nm to balance the loss of sensitivity for both analytes can be used if both these compounds are to be determined simultaneously [9].

The optimum solvent mixture, composed of acetonitrile–1% acetic acid containing 0.2% TEA (35:65), provided excellent baseline separation on a Hypersil® CN column. No significant alterations in peak shape and retention times were observed with a column having been used continuously for at least 1 month ($n > 500$). The addition of organic modifiers, such as TEA, to the mobile phase systems has been shown to be effective in reversed-phase HPLC for the separation and elution of amines and related ammonium compounds [1]. In this study, the addition of the antitailing agent, TEA, to the mobile phase greatly improved greatly improved the sensitivity of the method, because of increased peak sharpness as well as shortened retention times for propranolol and pronethalol.

Fig. 2 illustrates the different chromatograms observed as a result of evaluating three commercial HPLC CN columns. As seen in the figure, marked differences in retention times and resolution of the two drug peaks (I and II) in plasma were obtained. The Zorbax® CN (4.6 × 250 mm²; 5 μ; MacMod Analytical, Chadds Ford, PA) and Resolvex® CN

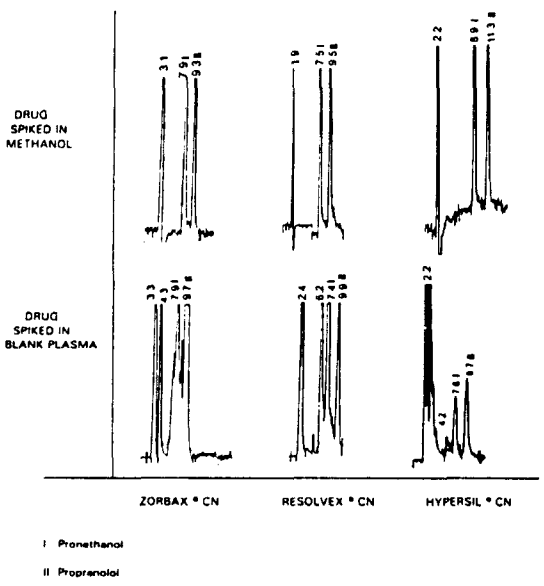


Fig. 2. Comparison of three commercial HPLC CN columns.

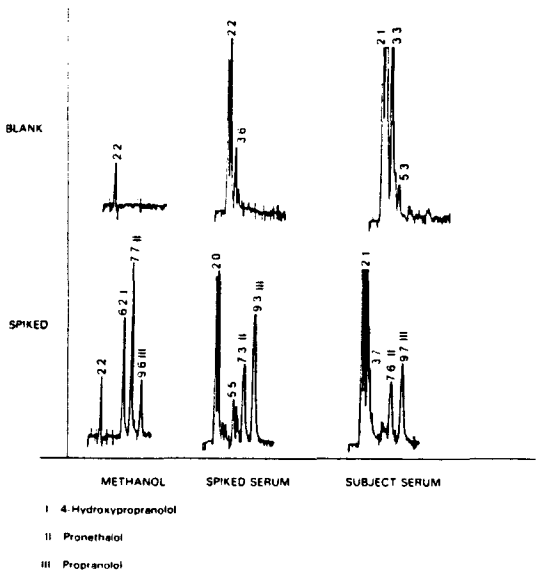
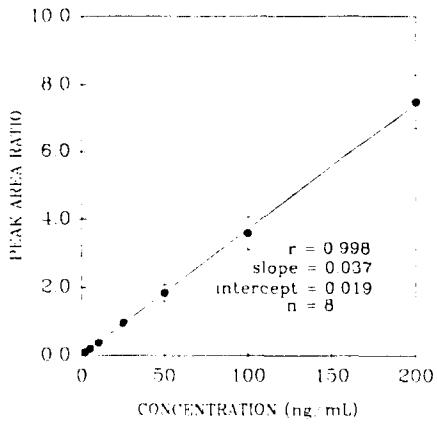


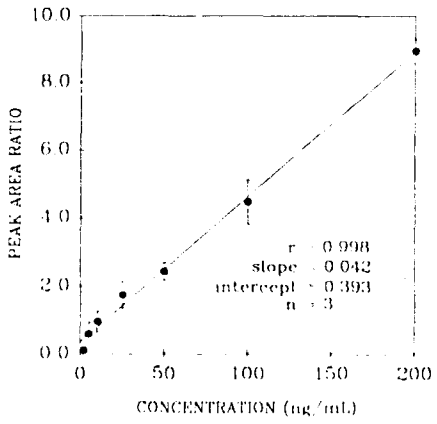
Fig. 3. Typical chromatograms.

(4.6 × 250 mm², 10 μ; Fischer Scientific, Fair Lawn, N.J.) columns showed significant interfering endogenous plasma peaks at about 4 min and about 6 min, respectively; however, both columns showed a good resolution when injected with pure methanolic standards. The Hypersil® CN (Alltech Associated, Deerfield, IL) column, on the other hand, bave a good baseline separation for the two drug peaks (II and III, $Rs = 1.92$), with elution in about 10 min, both from pure methanol and spiked serum/plasma. Additionally, this column showed a good column-to-column reproducibility, with no significant differences in retention times and resolution among the peaks. The blank chromatograms yielded no interfering peaks from endogenous plasma components, as shown in Fig. 3, represented by typical blank and drug spiked chromatograms in methanol, serum and subject serum respectively. Furthermore, 4-hydroxypropranolol, pronethalol, propranolol are all separated from each other, with approximate retention times of 6.5 min, 7.5 min and 9.5 min, respectively.

The linearity of the calibration curve in terms of the peak area ratio of each concentration relative to the internal standard in human serum and plasma are shown in Figs. 4a and 4b, respectively. The linearity of the assay was demonstrated by multiple analyses of the standard curves in serum ($n = 8$) and plasma ($n = 3$), indicating good linearity and reproducibility with a correlation coefficient $r^2 > 0.99$ (Table 1) in the range 1–40 ng of



(a)



(b)

Fig. 4. (a) Standard curve for propranolol in human serum; (b) standard curve for propranolol in human plasma.

propranolol on-column. This corresponds to 5–200 ng ml^{-1} of propranolol in 200 µl of serum/plasma. The slopes approached unity, indicating negligible proportional error in the assay, and the intercepts were not significantly different from zero, indicating negligible interference.

The inter-day (between-day) and intra-day (within-day) presision and accuracy of the method at five different concentrations of propranolol in serum are summarized in Table 2. The low relative standard deviation (RSD), which ranged from 4.8 to 6.6% demonstrated the good precision of the method. The mean accuracy for both inter-day and intra-day precision was found to be 99.4% of the actual value for the concentrations studied. Both the RSDs (below 15%) and the accuracy ($\pm$ 15%) of the method were within acceptable limits as recommended in the recent conference report on analytical method validation used for bioavailability, bioequivalence and pharmacokinetic studies [11]. The limit of quantitation (LOQ), the lowest concentration on the standard curve with acceptable precision RSD < 20%, was found to be 5 ng ml^{-1} and the limit of detection, the lowest concentration of propranolol that can be reliably differentiated from background levels, was found to be 2 ng ml^{-1} [11].

Table 1
Standard curve parameters for serum and plasma

Biological fluid	Slope	Intercept	r^2
Serum [a]	0.037 ± 0.004	0.019 ± 0.027	0.998
Plasma [b]	0.042 ± 0.004	0.393 ± 0.238	0.992

[a] Mean ± SD of 8 determinations.
[b] Mean ± SD of 3 determinations.

A variety of serum/plasma protein precipitants, namely ethyl acetate, methanol and hexane, were evaluated; however acetonitrile appeared to be the most appropriate. Similarly, a number of reconstituting fluids were evaluated; methanol yielded an excellent recovery of propranolol.

The analytical recovery of propranolol from both serum and plasma was determined at concentrations ranging from 2 to 200 ng ml^{-1}. Its mean recovery from serum/plasma ranged from 60.98 to 81.35%. This is not surprising. Because of its basicity ($pK_a = 9.5$) and high lipophilicity, propranolol has a greater tendency to adhere to glass surfaces; hence, the silanization of glassware was necessary. Furthermore, this also demonstrated the need for an internal standard (Table 3).

The specificity of the assay in serum/plasma was established by analyzing blank serum/plasma samples of various subjects ($n = 10$). There were no endogenous peaks that co-eluted with the drug peaks. Assay interference from other commonly used cardiac and other drugs was evaluated by injecting methanolic solutions of the drugs into the chromatographic system and recording their retention times. Drugs were considered non-interfering if their retention times were significantly different from those of 4-hydroxypropranolol, pronethalol and propranolol. Table 4 lists the drugs that were listed for potential interferences and their retention times.

The applicability of this analytical method has been demonstrated for the analysis of propranolol in order to study the pharmocokinetics of various experimental extended-release

Table 2
Inter-day and intra-day precision and accuracy of propranolol in human serum

Spiked concentration (ng ml^{-1})	Inter-day precision			Intra-day precision		
	Mean ± SD [a]	RSD (%)	Accuracy (%)	Mean ± SD [a]	RSD (%)	Accuracy (%)
5.0	4.8 ± 0.2	4.7	96.0	4.6 ± 0.2	4.8	92.0
10.0	10.3 ± 1.0	9.7	103.0	10.6 ± 0.7	6.3	106.0
25.0	25.8 ± 1.1	4.3	103.2	21.6 ± 2.1	9.7	86.4
50.0	49.6 ± 0.7	1.4	99.2	46.2 ± 3.6	7.7	92.4
100.0	98.1 ± 4.0	4.1	98.1	117.5 ± 5.3	4.5	117.5
		Mean 4.8	99.9		Mean 6.6	98.9

[a] Mean ± SD of 3 determinations.

dosage forms crossed over with a marketed immediate-release formulation (Inderal® IR 2 × 40 mg bid, Wyeth-Ayerst, NY) and a long-acting formulation (Inderal® LA 80 mg, Wyeth-Ayerst, NY) in six normal healthy volunteers (Fig. 5) [12].

4. Conclusions

Although a number of HPLC methods have been published for the determination of propranolol in serum/plasma, this reported method is an improvement over the published methods, and permits the rapid determination of propranolol separated from 4-hydroxypropranolol in serum/plasma. The preparation of serum/plasma samples, prior to chromatography, is relatively simple, and the total chromatographic run time is about 10 min. Standard curves for propranolol demonstrated good linearity over the range 5–200 ng ml^{-1}, with a limit of quantitation (LOQ) of 5 ng ml^{-1} and a detection limit of 2 ng ml^{-1}. The low (below 7%) intra-day and inter-day coefficients of variation at different concentrations demonstrate that the assay is accurate and reproducible. Finally, recoveries for both serum and plasma, ranged from 60 to 80%. It is concluded, therefore, that this analytical method is suitable for the routine clinical monitoring of serum/plasma levels in human subjects for bioequivalence studies and for use in pharmacokinetic research studies, using small laboratory animals where small aliquots of

Table 3
Recovery data of propranolol

Spiked concentration (ng ml^{-1})	Recovery (%)	
	Human serum [a]	Human plasma [b]
2.0	61.8	51.5
5.0	64.2	69.6
10.0	71.4	56.1
25.0	59.5	108.6
50.0	62.3	110.0
100.0	56.5	80.6
200.0	51.0	93.2
Mean	60.1	81.4
SD	6.4	23.7
RSD	10.5	29.2

[a] Average of 2 determinations.
[b] Average of 3 determinations.

Table 4
Drugs tested for potential interference in chromatography

Drug	Retention time (min)
Analytes	
4-Hydroxypropranolol	6.5
Pronethalol	7.5
Propranolol	9.5
Interfering	
Hydralazine hydrochloride	9.8
Ibuprofen	8.1
Isosorbide dinitrate	10.2
Nitroglycerin	10.1
Quinidine sulfate	9.1
Verapamil hydrochloride	10.0
Not Interfering	
Aspirin	2.9
Acetaminophen	> 12
Diltiazem hydrochloride	> 12
Dipyridamole	> 12
Furosemide	> 12
Hydrochlorothiazide	> 12
Procainamide hydrochloride	3.9
Theophylline	2.3
Phenylpropanolamine hydrochloride	> 12
Niacin	> 12
Nifedipine	> 12

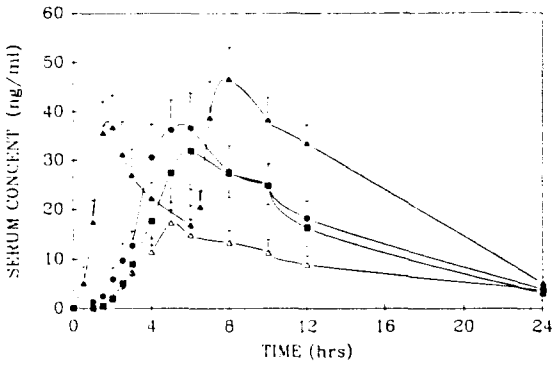


Fig. 5. Mean ± SEM serum propranolol concentrations for six human subjects. (●) Formulation 1; (■) Formulation 2; (△) Inderal® LA, 80 mg; (▲) Inderal® IR, 2 × 40 mg.

serum/plasma would permit the quantitation of propranolol.

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