

Development and validation of HPLC method for estimation of propranolol HCl in human plasma

M V Srikanth*, B Janaki Ram, S A Sunil, N Sreenivasa Rao and K V Ramana Murthy
A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003, India

Received 09 June 2011; revised 25 November 2011; accepted 28 November 2011

This study presents a rapid, sensitive and accurate high performance liquid chromatographic (HPLC) method for determination of propranolol HCl in human plasma. Human plasma samples were subjected to protein precipitation by methanol and protein free plasma samples were directly injected into HPLC C18 column. Mobile phase consisting of a mixture of acetonitrile: pH 4.5 phosphate buffer (35:65) was delivered at a flow rate of 1 ml/min. A good separation of propranolol HCl and diltiazem HCl as internal standard was achieved with retention times of 6.6 min and 9.9 min respectively. Linearity was obtained for propranolol (conc. range 20-280 ng/ml) and followed regression equation, $Y = 0.0162 X - 0.0515$. Peak areas were reproducible as indicated by low coefficient of variation ($< 2.13\%$).

Keywords: Accuracy, Human plasma, HPLC, Precision, Propranolol HCl

Introduction

Propranolol, a nonselective β -adrenergic receptor blocking agent possessing no other autonomic nervous system activity, is used for the treatment of hypertension. It is highly lipophilic and almost completely absorbed after oral administration¹⁻⁴. Among various methods developed for propranolol from biological matrices (serum, plasma, blood or urine), most of the methods⁵⁻⁸ require relatively large plasma volumes (> 1 ml), multiple steps of sample preparation and laborious time consuming extraction procedures. This study, approved from an independent Institutional Ethics Committee of Andhra University, Visakhapatnam, India, proposed a HPLC method, where human plasma (HP) samples were subjected to protein precipitation and protein free plasma samples were directly injected into HPLC column. Proposed method is simplified one-step sample preparation and can be adapted for routine pharmacokinetic studies of propranolol in clinical situations or small animal studies using a small amount (< 0.3 ml) of plasma.

Experimental Section

Materials

Propranolol HCl and Diltiazem HCl were provided by Dr Reddy's Laboratories Ltd (Hyderabad, India).

HPLC grade methanol and acetonitrile were purchased from Qualigens Fine chemicals Ltd, Mumbai, India. Potassium dihydrogen phosphate was purchased from S.D Chemicals, Mumbai, India. All other reagents and chemicals were of analytical grade.

A chromatogram system consisted of model SHIMADZU SPD M10 A VP series with LC solutions software. Samples were chromatographed at room temperature (25°C) on reverse phase C-18 column (3.9 mm x 300 mm, particle size 5 μm). A mixture of Acetonitrile: pH 4.5 phosphate buffer (35:65) was used as mobile phase (flow rate, 1 ml/min) and pressure was maintained at 90-150 kg/cm². Column temperature was maintained at 35°C . Mobile phase was filtered through 0.45 μm membrane filter before use. Ultraviolet absorption was measured at 214 nm using PDA detector.

Preparation of Stock Solution of Propranolol HCl

Propranolol HCl (50 mg) was dissolved in triple distilled (3D) water in a 100 ml volumetric flask and solution was made up to mark with the same to get 500 $\mu\text{g/ml}$. Of this stock solution, 1 ml was diluted up to 100 ml with 3D water to get 5000 ng/ml (Stock -I). Quantities (0.4, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8 and 5.6 ml) of stock -I solution were transferred to 10 ml volumetric flasks and were made up with 3D water to get 200, 400, 800, 1200, 1600, 2000, 2400 and 2800 ng/ml concentrations respectively. An aliquot 0.1 ml of 200, 400, 800, 1200,

*Author for correspondence
E-mail: venkatasrikanth_meka@yahoo.com

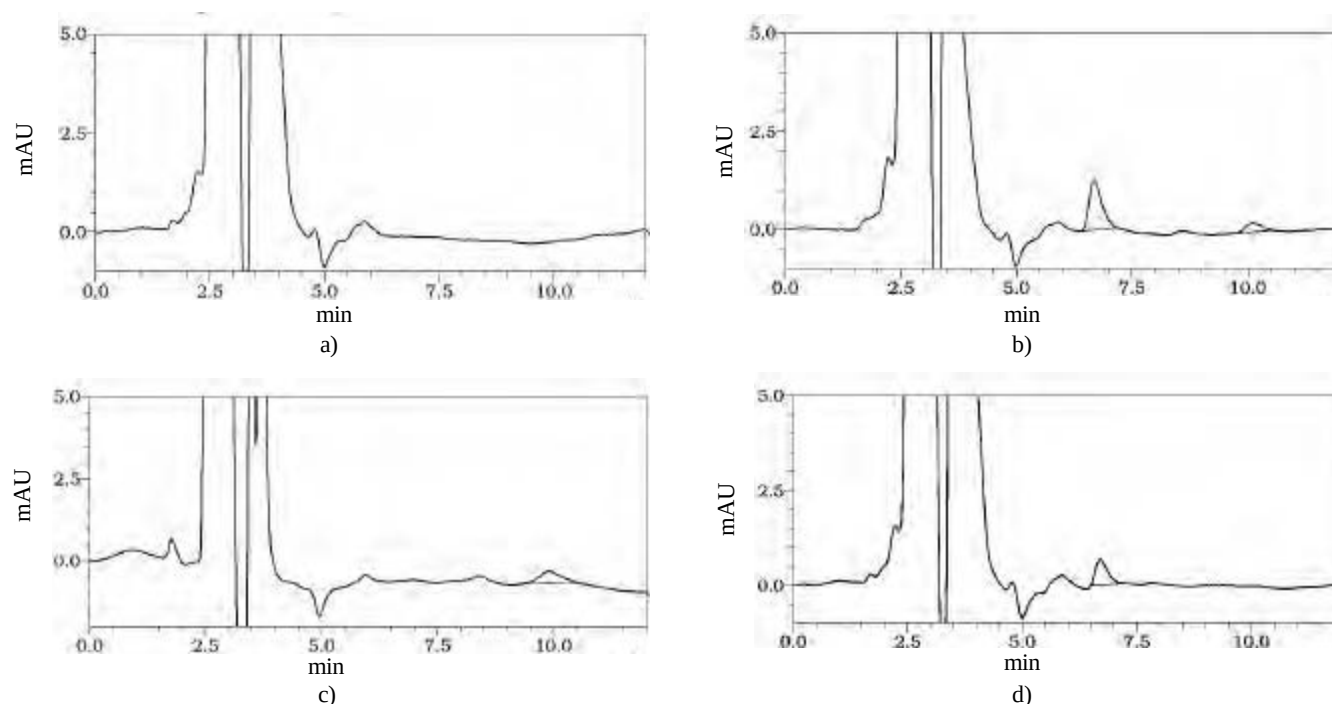


Fig. 1—Chromatograms: a) Blank human plasma; b) propranolol HCl in human plasma 200 ng/ml with internal standard; c) Internal standard without propranolol HCl; and d) Propranolol HCl without internal standard

Table 1—Concentration vs peak area ratios of propranolol HCl to internal standard in human plasma (n=3)

S.No	Concentration of propranolol HCl ng/ml	Peak area ratios of drug to internal standard	CV %
1	20	0.2518	0.55
2	40	0.5055	0.97
3	80	1.2523	1.22
4	120	1.9000	1.06
5	160	2.5333	0.45
6	200	3.2752	2.13
7	240	3.8502	1.99
8	280	4.4400	0.41

1600, 2000, 2400 and 2800 ng/ml represent 20, 40, 80, 120, 160, 200, 240 and 280 ng/ml respectively.

Preparation of Stock Solution of Internal Standard

Diltiazem HCl was used as an internal standard for estimation of propranolol HCl. Diltiazem HCl (50 mg) was dissolved in 3D water in a 100 ml volumetric flask and solution was made up to mark with the same to get 500 µg/ml. Of this stock solution, 1 ml was diluted up to 100 ml with 3D water to get 5000 ng/ml. An aliquot 0.1 ml of this solution represents 500 ng/ml.

Procedure

Blood sample (5 ml) was collected from ethical committee approved healthy volunteer and transferred into K₃ EDTA tubes. Sample was centrifuged at 7500 rpm for 10 min. Aliquots of HP samples (0.3 ml) were transferred into eppendorf micro-centrifuge tubes, and 0.1 ml each of propranolol HCl solutions and internal standard solution, and 0.5 ml of methanol were added. Mixture of samples was vortexed for 3 min using Remi cyclo mixer followed with centrifugation for 20 min at 7500 rpm. Supernatants were collected and 20 µl of samples were directly injected to HPLC column by using micro syringe. Chromatogram quantification was performed using peak area ratios of propranolol HCl to internal standard (Table 1, Fig. 1). Separation process of HPLC instrument can be done by reversed phase HPLC system, which was characterized by strong interaction between sample molecules and polar mobile phase, due to dipole interaction or hydrogen bonding of solvents basicity (proton acceptor) or solvent acidity (proton donor).

Precision and Accuracy⁹

Intra-day (within-run) and inter-day (between-run) precision and accuracy of present HPLC method were estimated by subjecting propranolol HCl standard

Table 2—Precision and accuracy of HPLC method used for estimation of propranolol HCl (n=5)

Target	Propranolol HCl conc., ng/ml		CV		RE	
	Measured		%		%	
	(Mean, n=5)					
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
80	78.65	79.23	1.51	1.96	-1.68	-0.96
120	119.09	120.12	1.04	1.16	-0.76	0.1
200	196.95	197.09	1.18	1.32	-1.5	-1.45

solutions 80, 120 and 200 ng/ml to analysis. Intra-day precision and accuracy were determined by assaying HP samples of five each for each concentration within one day, whereas inter-day precision and accuracy were determined by assaying five samples for each concentration for five consecutive days. In each case, coefficient of variation (CV%) in peak area of drug was calculated to find precision and relative error (RE%) was determined to find accuracy of present HPLC method (Table 2).

Recovery⁹

Recovery of an analyte in an assay is the detector response obtained from an amount of analyte added to and extracted from biological matrix, compared to detector response obtained for true concentration of pure authentic standard. Recovery pertains to extraction efficiency of an analytical method within the limits of variability. Recovery of precipitation procedure was conducted by adding 80, 120, 200 ng/ml of propranolol HCl to pre-analyzed plasma drug samples containing 80 ng/ml of propranolol HCl and subjected them to present HPLC method. Five (n=5) spiked plasma samples at these different concentration levels were subjected for analysis to calculate mean recovery. All spiked plasma samples contained the IS at same concentration level.

Results and Discussion

Method Development

A number of HPLC methods reported were evaluated for analysis of propranolol HCl in HP, but none provides satisfactory results in terms of processing and reproducibility¹⁰⁻¹⁴. To obtain good separation between propranolol HCl and internal standard (Diltiazem HCl), different types of columns and mobile phases were examined. Diltiazem HCl, due to its structural similarity to propranolol HCl, was chosen as the IS to normalize erratic recoveries and to improve precision of analysis.

Among plasma extraction methods, protein precipitation method with methanol was found to be optimal, and produced a clean chromatogram for a blank HP sample and yielded highest and stable recovery for analyte from the plasma. Chromatographic conditions, especially composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. Finally, a mixture of acetonitrile: pH 4.5 phosphate buffer (35:65) as mobile phase (flow rate, 1 ml/min) and pressure maintained at 90-150 kg/cm² was optimized as chromatographic conditions. A good separation of propranolol HCl and diltiazem HCl as internal standard was achieved with retention times of 6.6 min and 9.9 min respectively without any interference of endogenous compounds in HP. Each sample was injected in three times and observed same retention times in all cases. A representative standard curve of propranolol HCl- internal standard peak area ratio over propranolol concentration range 20-280 ng/ml resulted in the following linear least squares regression equation: $Y = 0.0162 X - 0.0515$, where X is concentration of propranolol HCl (ng/0.3 ml HP) and Y is the ratio of peak areas of propranolol HCl to internal standard. A good linear relationship was observed as indicated by $r = 0.9997$. Peak areas were reproducible as indicated by low coefficient of variation (< 2.13%).

Accuracy and Precision

HPLC method was highly precise, which was confirmed by low coefficient of variation (< 1.96%) in inter- day as well as intra- day estimation of propranolol HCl (Table 2). RE% values were found to be within the limits of ± 1.68 , indicating high accuracy of present HPLC method (Table 2).

Recovery

For different concentrations (ng/ml), recovery (n=5) of propranolol HCl from preanalysed HP drug solution

are as follows: 80, 99.59 ± 0.99 ; 120, 99.97 ± 0.94 ; and 200, $100.03 \pm 0.17\%$. Recovery results demonstrated good efficiency (detection of $> 99\%$ of propranolol HCl) of precipitation procedure employed in this study. In a HPLC method for estimation of propranolol HCl in HP, developed by Gurvinder *et al*¹⁵, excess use of organic solvents and high flow rate is not economical. Jing Zhang *et al*⁸ developed HPLC method using 0.5 ml of plasma for determination of propranolol in HP but method involved complicated process for plasma extraction along with more number of organic solvents for sample preparation, which is expensive and leads to high production cost for analysis of regular samples. Thus present HPLC method is more precise, rapid and sensitive.

Conclusions

Proposed method has a good sensitivity and specificity for determination of propranolol in HP. A good separation of propranolol HCl and internal standard was achieved without any interference of endogenous compounds in HP. Standard curve for propranolol HCl demonstrated that good linearity over the range of 20-280 ng/ml. The low intra-day and inter-day coefficients of variation at different concentrations demonstrated that assay is accurate and reproducible. Thus present HPLC method is suitable for routine clinical monitoring of plasma levels in human subjects for bioequivalence studies and for use in pharmacokinetic research studies, using small laboratory animals where small aliquots of HP would permit quantitation of propranolol HCl.

Acknowledgements

Author (M V Srikanth) thanks UGC, Govt of, India, New Delhi for awarding JRF for carrying out this project, and also thankful to Mr K Praveen Kumar and Mr C Vasu for providing valuable information to carry out this research work.

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