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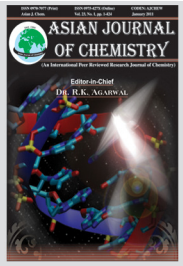

High-Performance Liquid Chromatography Determination of Propranolol in Human Plasma

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A simple rapid, selective sensitive and novel high pressure liquid chromatographic (HPLC) method was developed and validated for the assay of propranolol. Sample preparation was performed by liquid-liquid extraction in ethyl acetate. The organic solvent was evaporated and the residue dissolved in a mobile phase. Aliquots of 60 µL were injected automatically into the chromatograph. The separation of propranolol was achieved on a reversed phase C_{18} column with a mobile phase consisting of 10 mM ammonium acetate, acetonitrile,: TEA (70:30:0.01 v/v/v). Quantitation of propranolol was performed by UV detection at 210 nm. The linearity range was 5 to 80 ng/mL. Inter-day and intra day accuracy and precision was found 91.26-109.13, 2.79-5.79 and 92.15-108.52, 1.52-5.67 respectively. Long term stability for 30 days of propranolol in human plasma was proved as per guidelines.

Key Words: High performance liquid chromatography, Propranolol.

INTRODUCTION

Propranolol, a β-adrenergic antagonist, is used to treat angina pectoris, cardiac arrhythmias and hypertension. 4-Hydroxy propranolol, a metabolite of propranolol in humans and animals[1], is equipotent with propranolol as a β-adrenergic antagonist[2] and is present in human plasma after acute and chronic treatment with propranolol[3-7].

Several method for the estimation of propranolol have been proposed, including mass fragmentography[3] direct fluorometry[4] and reverse phase HPLC with fluorescence detection[5], but none of these methods is entirely satisfactory.

This paper describes a specific and sensitive high-performance liquid chromatography method for the determination of propranolol in plasma with UV detection[8]. Our aim is to develop a general method for each agent. Validation studies for the propranolol were performed with respect to recovery, selectivity, linearity, precision and accuracy[9]. The procedure has been used for the analysis of plasma samples of patients taking propranolol.

EXPERIMENTAL

Propranolol provided from Raptim Research Ltd. and supplies also the COA of that drug. All reagents were of analytical grade. Ammonium acetate (Rankem), *tert*-butyl methyl ether (Rankem), hydrochloric acid (Merck) and acetonitrile (HPLC, Rankem). Milli-Q water, millipore water was used for the preparation of buffer and other aqueous solutions. Extraction method is liquid-liquid extraction.

Chromatographic separation of the propranolol was performed on a Merck, purosphere C_{18} column (150 mm × 4.6 mm *i.d.*). The HPLC equipment comprized of a solvent delivery system (Agilent 1100 series) and UV detector. The auto ampler (Agilent) was used to sample injection and the column was kept in oven (Agilent), UV detection was performed at 210 nm. Chemstation software was used for data analysis. A Lab India pH meter was used to measure the pH of the aqueous mobile phase. The mobile phase was delivered at an isocratic rate of 1.5 mL/min with a pump pressure of approximately 150 bar. Total run time for plasma samples was 10 min.

Chromatographic conditions: The separation of propranolol was achieved on a reversed phase C_{18} column with a mobile phase consisting of 10 mM ammonium acetate: acetonitrile: TEA in the ratio 70:30:0.01 % (v/v/v). Quantitation of propranolol was performed by UV detection at 210 nm, at CTO 40 ºC and flow 1.5 mL/min.

Standard and stock solution preparation: Stock solution of propranolol prepared in methanol (1000 ppm), weigh 25 mg of propranolol transfer to volumetric flask, add 10 mL of methanol sonicate it, adjust the volume with methanol. Solutions were stored at 2-8 ºC was used to spike plasma samples. Standards and quality control samples were made by addition of the determined quantity of stock solution to drug free plasma and

stored at -20 ºC in aliquots. Plasma quality control sample concentrations were 15, 40 and 70 ng/mL

Preparation of plasma samples: Take 0.5 mL of plasma in ria vial, add 100 µl of 0.1 N HCl, vortex the vials on vortexer, then add 3 mL of *tert*-butyl methyl ether, vortex vials for 3 min on vortexer, then centrifuge the vial in cold centrifuge for 10 min at 4500 rpm on 4 ºC. Separate 2 mL of upper layer of organic solvent in vial, evaporate to dryness at 50 ºC under a steady stream of nitrogen. Following reconstitution of the residue in 100 µL of mobile phase, the mixture was transferred to a micro insert and 60 µL was then injected on to HPLC.

Calibration and linearity: Assay performance was determined in accordance with FDA guidance for bioanalytical method validation for human studies. Standard curve were constructed using seven standards non-zero concentrations in plasma and run in duplicate daily for three consecutive days. Drug concentrations were reported as the area of drug. In plasma the standard concentrations range for 5 to 80 ng/mL. Calibration curves were generated using weighted least squares regression analysis and obtained over the respective standard concentrations in plasma. All standards and quality control samples were stored at -20 ºC until analysis.

Precision and accuracy: Standards and replicate quality control samples of plasma at each concentration were analyzed on three consecutive days, after, which inter-and intra-day means, % nominal and coefficients of variation (% CV) were calculated by standard method.

Validation parameters: Validation parameters are tested according to guidelines for the proposed method. Stability of propranolol was evaluated according to the freeze thaw cycle, refrigerator (holding time) and auto sampler stability. In FTC test plasma samples was stored in -20ºC temperature. Samples were taken out from the deep freezer and equilibrated to room temperature and analyzed. The results were compared with fresh CC samples. Refrigerator stability of propranolol in plasma was evaluated in spiked samples. Samples were taken out from refrigerator after 5 days and equilibrate to room temperature and analyzed. The results were comparing with fresh CC samples. Auto sampler stability of propranolol in the reconstituted plasma samples were evaluated for 36 h. The calibration curve consists of seven concentration levels of spiked standards of lower limit of detection 5 ng/mL. The result were calculated in 1/C weighted condition. The linear calibration curve between the area and the concentration of the target propranolol was established by linear regression.

The limit of detection values were calculated by using a signal-to-noise ratio of 3.2, while limit of quantification value was calculated by using a signal-to-noise ratio of 10. To assess intra-day precision, six spiked samples at three concentration levels quality control samples (15.00, 40.00 and 70.00 ng/mL) were prepared and analyzed.

Recovery experiments were performed by comparing the analytical results of extracted standard samples with plasma samples. This experiment was performed on quality conterol samples

Biological application: To demonstrate the applicability of the procedure for measurement of propranolol levels in pharmacokinetics studies on healthy male volunteers.

RESULTS AND DISCUSSION

To develop a sensitive, selective and simple assay method for the extraction and quantification of propranolol during method development different options were evaluated to optimize detection and chromatography parameters. First we have to find out the wavelength using the references. Then select the mobile phase varying the composition and pH, optimize it. Further optimization in chromatography conditions increased the response of analyte. A mobile phase containing 10 mM ammonium acetate buffer in combination with acetonitrile resulted in improved response. Use of purosphere C_{18} 150 × 4.6 mm × 5 µm) column resulted in HPLC run time of 7 min.

Selectivity: Selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times of drug in six different lots of extracted blank plasma. Representative chromatographs of extracted blank plasma, extracted plasma samples containing 20 ng/mL of propranolol are given in Figs. 1, 2a and 2b.

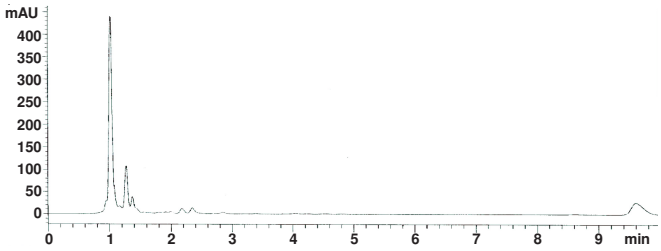

Fig. 1. Chromatograph of blank sample there is absence of endogeneous material at the RT of drug

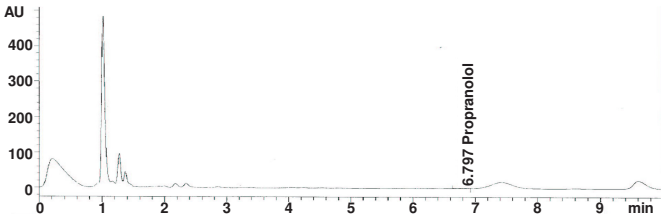

Fig. 2a Lower limit of quantitation of propranolol

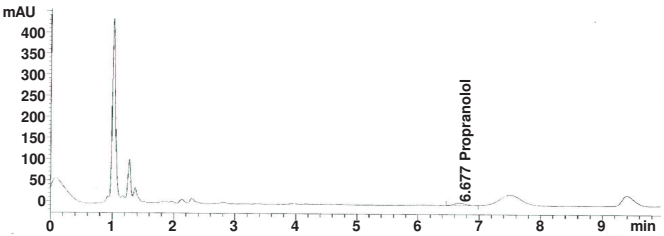

Fig. 2b. Higher limit of quantitation of propranolol

Linearity and lower limit of detection: The peak area of calibration standards was proportional to the concentration of analyte in each assay over the nominal concentration range of 20.00 to 640.00 ng/mL for propranolol. The calibration curves appeared linear and were well described by least squares lines. A weighting factor of 1/X concentration was chosen to achieve homogeneity of variance. The correlation coefficients were = 0.99 (n = 4) for propranolol. The mean (± S.D.) slopes of the calibration curves (n = 4) for propranolol were 0.1830

(± 0.0166). Results of four representative standard curve for HPLC determination of propranolol are given in Table-1. The lower imit of quantification for propranolol proved to be 20 ng/mL.

TABLE-1
RESULTS OF FOUR CALIBRATION CURVES FOR DETERMINATION OF PROPRANOLOL IN HUMAN PLASMA

Actual Concentration	Area	Calculated Concentration	% Nominal
5.00	5.62	5.62	112.42
10.00	10.41	10.41	104.11
20.00	20.73	20.73	103.66
30.00	28.82	28.82	96.07
45.00	44.03	44.03	97.85
60.00	58.68	58.68	97.80
80.00	81.70	81.70	102.13
Slope	–	1.000	–
Intercept	–	-0.001	–
Regression	–	0.9982	–

Precision and accuracy: Intra-batch and inter-batch precision and accuracy of the method for propranolol are presented in Tables 2 and 3. The precision deviation values for intra-batch and inter-batch are all within 15 % of the relative standard deviation (RSD) at each quality control level. The accuracy deviation values for intra-batch and inter-batch are all within the (100 ± 15) % of the actual values at each quality control level.

TABLE-2
INTRA BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF PROPRANOLOL IN HUMAN PLASMA

PA Batch	LQC	MQC	HQC
	15 ng/mL	40 ng/mL	70 ng/mL
1	16.85	35.25	59.65
2	15.93	36.61	64.92
3	16.12	35.83	61.07
4	16.04	39.36	64.18
5	17.03	37.78	70.28
6	16.25	37.87	63.18
7	16.45	41.53	65.43
8	15.90	44.20	61.35
9	13.75	42.34	61.07
10	13.45	39.36	70.43
11	14.35	40.65	70.28
12	16.25	42.45	75.38
13	15.03	41.76	67.32
14	16.00	41.15	70.67
15	16.23	38.75	76.85
16	13.85	40.35	70.65
17	14.65	43.54	73.67
18	15.75	41.37	70.43
Average	15.14	41.45	69.46
SD	1.09	1.58	4.95
%CV	7.22	3.81	7.13
% Nominal	100.92	103.63	99.23

Recovery: Results of extraction efficiency measured for propranolol was consistent, precise and reproducible. The mean related extraction recovery of propranolol at each quality control level (60, 300 and 600 ng/mL) was 68.05, 66.40 and 64.04 %, respectively.

TABLE-3
THE INTER BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF PROPRANOLOL IN HUMAN PLASMA.

PA Batch	LQC	MQC	HQC
	15 ng/mL	40 ng/mL	70 ng/mL
1	16.85	35.25	59.65
2	15.93	36.61	64.92
3	16.12	35.83	61.07
4	16.04	39.36	64.18
5	17.03	37.78	70.28
6	16.25	37.87	63.18
7	16.45	41.53	65.43
8	15.90	44.20	61.35
9	13.75	42.34	61.07
10	13.45	39.36	70.43
11	14.35	40.65	70.28
12	16.25	42.45	75.38
13	15.03	41.76	67.32
14	16.00	41.15	70.67
15	16.23	38.75	76.85
16	13.85	40.35	70.65
17	14.65	43.54	73.67
18	15.75	41.37	70.43
Average	15.55	40.01	67.60
SD	1.09	2.59	5.22
%CV	7.02	6.48	7.72
% Nominal	103.66	100.02	96.57

Stability: Results of autosampler stability, freeze thaw stability, long term stability are given in Tables 4-7. Stability data gives the idea about reliable stability of propranolol in tested conditions. Propranolol was found stable during short term and long term stock solution stability. During short term stock solution stability % mean stability of propranolol was found 99.54 %. Long term stock solution stability was performed for 6 days and % mean stability of propranolol was found 100.05 %.

TABLE-4
BENCH TOP STABILITY (FOR 7 h)

Sr. No.	Bench top stability			
	LQC (15 ng/mL)		HQC (15 ng/mL)	
	Fresh	Stability	Fresh	Stability
1.	13.48	16.08	64.24	78.18
2.	14.49	14.06	61.84	71.58
3.	12.99	13.93	72.21	75.08
4.	13.03	15.02	65.02	72.00
5.	14.00	15.35	67.54	71.54
6.	12.98	14.52	63.25	71.25
Average	13.495	14.827	65.683	73.272
SD	0.630	0.821	3.722	2.790
%RSD	4.67	5.54	5.67	3.81
% Nominal	89.97	98.84	93.83	104.67
%Difference (B-A) A*100	9.87		11.55	

TABLE-5
SHORT TERM STABILITY IN MATRIX (FOR 7 DAYS)

Sr. No.	LQC 15 ng/mL	MQC 40 ng/mL	HQC 70 ng/mL
1	16.34	40.25	70.32
2	15.27	41.62	64.25
3	16.54	44.31	68.24
4	17.01	43.21	72.48
5	14.26	42.01	73.21
6	14.50	39.62	71.65
Average	15.65	41.84	70.03
SD	1.14	1.76	3.33
% RSD	7.29	4.21	4.76
% Nominal	104.36	104.60	100.04

TABLE-6
AUTO SAMPLER STABILITY (FOR 24 H)

Sr. No.	LQC 15 ng/mL	MQC 40 ng/mL	HQC 70 ng/mL
1.	15.00	36.25	75.24
2.	16.23	38.54	72.15
3.	16.86	37.66	73.26
4.	15.33	39.85	71.42
5.	17.00	37.25	70.28
6.	15.86	37.87	73.24
Average	16.05	37.90	72.60
SD	0.81	1.22	1.72
% RSD	5.02	3.21	2.37
% Nominal	106.97	94.76	103.71

TABLE-7
FREEZE THAW CYCLE (AFTER 3 FTC)

Sr. No.	LQC 15 ng/mL	MQC 40 ng/mL	HQC 70 ng/mL
1	17.21	40.01	70.21
2	13.24	42.32	68.23
3	15.24	45.01	64.12
4	16.04	44.22	72.48
5	17.24	41.05	75.41
6	16.25	38.25	71.65
Average	15.87	41.81	70.35
SD	1.49	2.56	3.88
% RSD	9.41	6.13	5.51
% Nominal	105.81	104.53	100.50

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