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Development of a Validated HPLC Method for the Estimation of Metformin HCl and Propranolol HCl

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Authors' contributions

This work was carried out in collaboration between all authors. Author VSM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JRB and SRD managed the analyses of the study. Author AG managed the literature searches. Author VRMK supervised the work. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

The present investigation targets to develop a simple, specific, sensitive and accurate reverse phase high performance liquid chromatographic (RP-HPLC) method in human plasma for the estimation of metformin HCl and propranolol HCl from bulk drug and also from the marketed products. Human plasma samples were subjected to correct procedure for protein precipitation by methanol and protein free plasma samples were directly injected into HPLC C18 column. Chromatographic determination was performed on a reversed phase C18 column (3.9 mm X 300 mm, particle size 5 µm) using a mixture of acetonitrile and 0.1M pH 4.5 potassium dihydrogenortho phosphate buffer (mL) (40:60) at a flow rate of 0.8 mL/min and maintained at a pressure of 140 to 150 Kg/cm^2. The retention time for metformin HCl and propranolol HCl was found to be 9.084 min and 6.132 min respectively at 232 nm without any interference of endogenous compounds in

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the plasma. The method was linear in the range between 50-2000 ng/mL. The peak areas were reproducible as indicated by low coefficient of variation. It was found that the excipients in the tablet dosage form do not interfere in the quantification of active drug by proposed method.

Keywords: Metformin HCl; propranolol HCl; validation; HPLC method; human plasma; accuracy; precision.

1. INTRODUCTION

Now days, the trend goes towards the development of a single analytical method for the estimation of individual drug from a multidrug composition and it's a great challenge to the researchers. Single analytical method for different drugs is very advantageous to the industry as it is economic, avoids the wastage of chemicals and less time consuming [1]. In the present research, author targets to develop a simple, specific, sensitive and accurateanalytical RP-HPLC method in human plasma for the estimation of metformin HCl and propranolol HCl from bulk drug and also from pharmaceutical dosage form.

Metformin HCl is a biguanide anti hyperglycemic agent used in the management of non-insulin dependent diabetes mellitus (type II diabetes) [2]. Metformin in pharmacological doses does not reduce basal blood glucose concentration below the physiological range in either diabetic or non-diabetic animals or humans, a distinct advantage over sulfonylureas. Hence, it is considered as an antihyperglycemic agent rather than a hypoglycemic agent [3]. Metformin is a high polaric biguanide compound and it is very difficult to make extract from the from the biological samples using organic solvents [4].

Propranolol is a beta-adrenergic blocker used for the treatment of hypertension. It is highly lipophilic and almost completely absorbed after oral administration [5-6].

Literature survey revealed that different methods have been developed for the estimation and quantification of metformin HCl as well as propranolol HCl from the biological fluids such serum, plasma and urine and reported their own advantages [7-12].

Porta V et al. [7] developed a simple, rapid and sensitive HPLC method for determination of metformin in plasma samples from bioequivalence assays. Protein precipitation with acetonitrile was employed for the sample preparation. Chromatographic separation was performed on a reversed-phase phenyl column at 40ºC and the wavelength was set at 236 nm. The method was allowed the determination of metformin at low concentrations with a higher throughput than previously described methods.

A simple, accurate, economical and reproducible HPLC method has been developed by Mousumi et al. [8] for quantitative estimation of metformin hydrochloride from tablet dosage form and from formulated microspheres. The mobile phase used in the investigation was acetonitrile: phosphate buffer at the ratio of 65:35 and the pH adjusted to 5.75 with orthophosphoric acid. Glipizide is used as internal standard. The linearity was observed in concentration range of 0-25 µg/ml for metformin hydrochloride. Results of analysis were validated statistically and by recovery studies.

Most of the reported analytical methods require relatively large plasma volumes (more than 1 ml), multiple steps of sample preparation and laborious time consuming extraction

procedures [13-15]. Even though, many reported methods are available with their own advantages, they still do need of large plasma volumes and are not frugally possible for repetitive use in bioavailability studies. As stated before, single analytical method for different drugs can minimize the cost and also time benefit. Hence, studies were carried out to develop a simple HPLC method for the estimation of metformin HCl and propranolol HCl in plasma samples. The method employed in this study involved the direct injection of the plasma sample after protein precipitation without resorting to the evaporation techniques.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Metformin HCl and propranolol HCl bulk samples were provided by Dr Reddy's Laboratories Ltd (Hyderabad, India) (Purity of the compounds are 99-101%). Metformin HCl commercially available tablets 500 mg; (Glycomet 500 SR, 500 mg, B. No. 28006536) USV Ltd., India, purchased from the local market. Propranolol HCl commercially available tablets 80 mg: (Ciplar LA 80, 80 mg, B. No. D80624), manufactured by Cipla Ltd, India. Methanol and acetonitrile in HPLC grade were purchased from Qualigen Fine chemicals Ltd, Mumbai, India. Potassium dihydrogenortho phosphate was obtained from S.D Chemicals, Mumbai, India. All other reagents and chemicals were of analytical grade available in our laboratory.

2.2 Instrumentation and Chromatographic Conditions

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(3.9 mm X 300 mm, particle size 5 µm) column. The mobile phase consisting of acetonitrile: 0.1M pH 4.5 potassium dihydrogenortho phosphate buffer (40:60) at a flow rate of 0.8 mL/min and maintained at a pressure of 140 to 150 Kg/cm^2. The mobile phase was filtered through 0.45 µm filter and degassed by ultrasonication before use. Metformin HCl and internal standard was measured at a wavelength of 232 nm using Shimadzu SPD M10 AT VP model Photo Diode Array (PDA) detector. The HPLC system was equipped with "LC-solutions (Shimadzu Corporation, Switzerland)"software.

2.3. Calibration Curve

2.3.1 Preparation of stock solution of metformin HCl and propranolol HCl

Stock solutions were prepared by dissolving 100 mg each of Metformin HCl and propranolol HCl in triple distilled (3D) water in a 100 mL volumetric flask separately and the final volume was made up to the mark with 3D water to get 1000 µg/mL solution (Stock-I). 5 mL of this Stock-I solution was diluted up to 100 mL with 3D water to get 50 µg/mL solution (Stock–II). 0.1, 0.2, 0.4, 1, 2 and 4 mL quantities of the Stock –II solution was diluted to 10 mL with 3D water to get the standard solutions containing 500, 1000, 2000, 5000, 10000 and 20000 ng/mL concentrations of metformin HCl as well as propranolol HCl. An aliquot of 0.1 mL of the above standard concentrations represents 50, 100, 200, 500, 1000 and 2000 ng/mL of metformin HCl as well as propranolol HClrespectively.

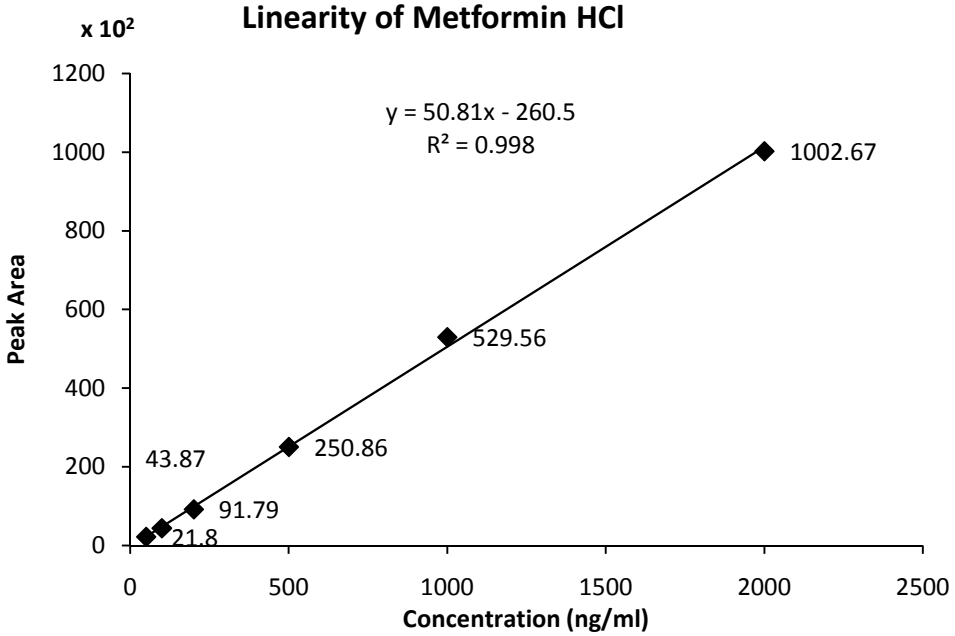
2.4 Procedure

5 ml of the blood sample was collected from ethical committee approved healthy volunteer and transferred into K_3 EDTA tubes. The collected blood sample was centrifuged at 7000 rpm for 10 min. 0.3 ml of aliquots of human plasma samples were transferred into eppendorf micro-centrifuge tubes, 0.1 ml each of metformin HCl and propranolol HCl solution, and 0.5 mL of 50:50 mixture of acetonitrile: methanol solution were added. Mixture of samples was vortexed for 3 minutes using Remi cyclomixer followed with centrifugation for 15 minutes at 7000 rpm.The supernatant protein free plasma was collected using micro pipette and directly injected into HPLC column by using 20 µL Hamilton micro syringe.

The present study was approved by independent Institutional Ethics Committee of Andhra University, Visakhapatnam (India).

2.5 Quantification

The quantification of chromatogram was performed by measuring peak area of the curve at particular concentration. The average values for 3 such determinations were measured and plotted a graph in-between the peak area vs. concentration (Fig. 1). The correlation coefficient (r) for each drug was calculated. Chromatograms obtained with blank human plasma and plasma spiked with drugs is shown in the Fig. 2.



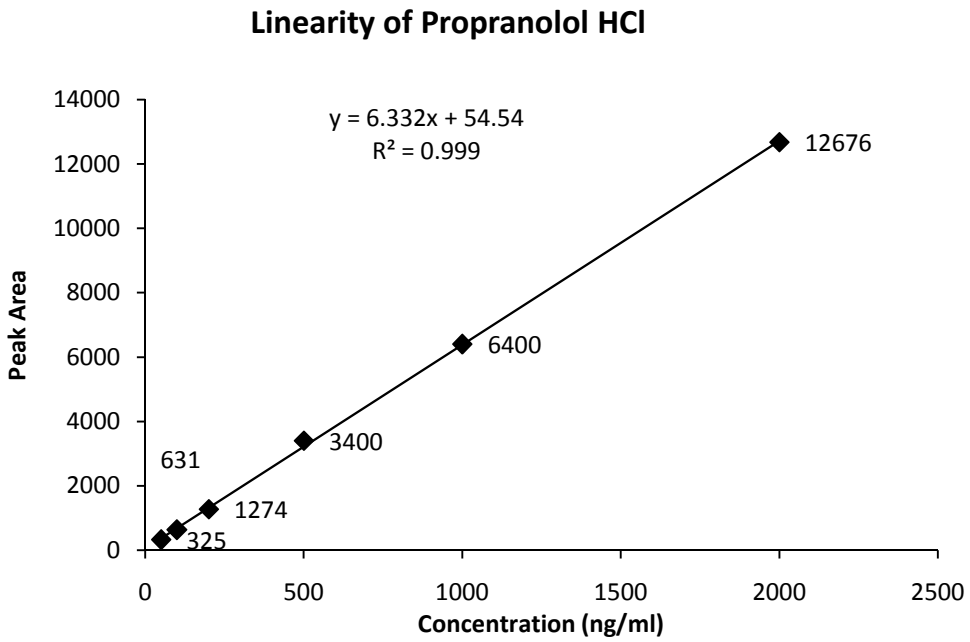


Fig. 1. Linearity graphs

2.6 Analytical Method Validation

2.6.1 Specificity

Specificity is the ability of the HPLC method to estimate response of the analyte in presence of its potential impurities. In the present investigation, specificity was performed to know the drug retention time in a mixture and in the individual sample to understand if any drug-drug interaction is present.

2.6.2 System suitability

The resolution and reproducibility adequateness of the chromatographic systems was verified by using system suitability. The tests are depends on the equipment, electronics and samples constitutes. The limits for system suitability were set for resolution, theoretical plates, and asymmetry.

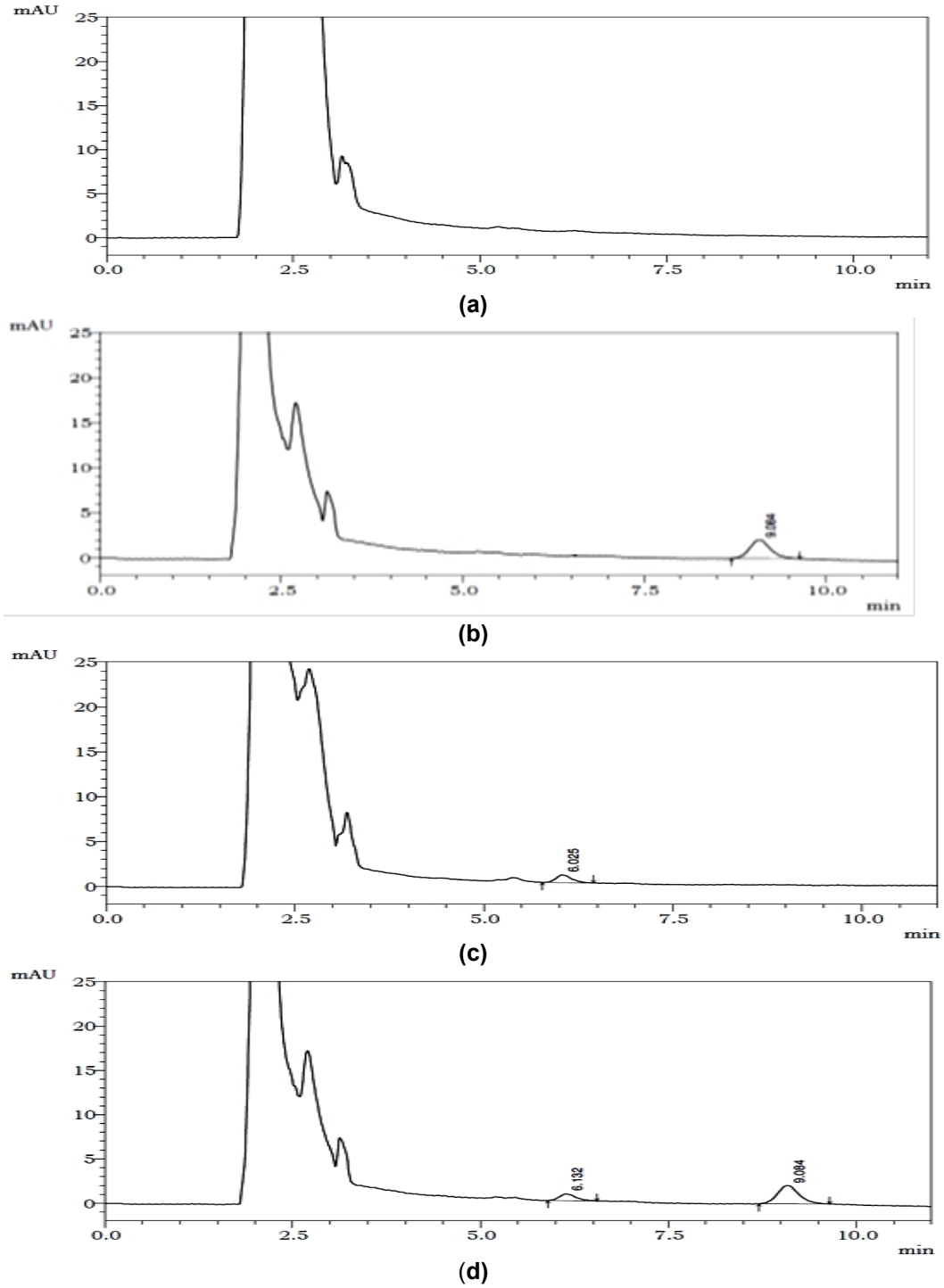


Fig. 2. Chromatograms obtained (a) blank human plasma; (b) plasma spiked with metformin HCl (200 ng/ml); (c) plasma spiked with propranolol HCl (200 ng/ml); (d) plasma spiked with metformin HCl and propranolol HCl (each 200 ng/ml)

2.6.3 Precision and accuracy [16]

The precision designates the closeness of individual reading of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte.

The intra-day (within run) and inter-day (between run) variation of the present HPLC method was estimated by subjecting 100, 200, 500 and 1000 ng/mL of metformin HCl standard solutions to analysis. Precision and accuracy were determined by assaying five samples for each concentration within one day for intra-day and on five consecutive days for inter-day. In each case the coefficient of variation (% CV) in peak area of the drug was calculated to find the precision and % relative error (% RE) was calculated to find out the accuracy of the present HPLC method.The same procedure was repeated for propranolol HCl. The results are given in the Table 2.

2.6.4 Recovery [17]

Recovery of the precipitation procedure was conducted by adding 100, 200, 500 and 1000 ng/mL of metformin HCl/propranolol HCl to the preanalysed plasma drug samples containing 100 ng/mL of metformin HCl/propranolol HCl and subjected them to the 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 same concentration level of internal standard.The data of the experiment were statistically analyzed using the formula [% Recovery = (Recovered conc./Injected conc.) x 100]. The results are given in Table 3.

2.6.5 Limit of detection and limit of quantification [18]

The limit of Detection (LOD) and limit of Quantification (LOQ) of the HPLC method were determined by increasingly injecting the lower concentrations of the standard solutions. The LOD is the least concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOQ is the smallest concentration of the analyte, which produces a response that can be accurately quantified (signal to noise ratio of 10).

2.6.6 Robustness

Robustness of the present analytical method was performed by altering the chromatographic conditions such as change in the mobile phase concentration and flow rate. In the present developed method, the mobile phase mixture contains acetonitrile and 0.1M pH 4.5 potassium dihydrogenortho phosphate buffer (40:60) and it has been changed to 30:70 & 50:50 to check the robustness. In the similar way the flow rate changed to 0.6 mL/min and 1.0 mL/min.

2.6.7 Ruggedness

Ruggedness of the method was conducted by carrying out the analysis on different days with different analysts to check for any changes in the chromatograph. The percentage RSD for the retention time and area was calculated.

2.6.8 Stability

The bench top stability was examined by keeping replicates of the low, medium and high plasma quality control samples at room temperature for approximately 24 h. Freeze-thaw stability of the samples was done by thawing at room temperature for 2-3 h, refrozen for 12-24 h. Auto sampler stability of metformin HCl and propranolol HCl was tested by the analysis of processed and reconstituted low, medium and high plasma samples, which are stored for 24 h at 5±1ºC. Stability of the metformin HCl and propranolol HCl in human plasma was also tested after storage at approximately 70±5ºC for 30 days. For each storage condition and each concentration, three replicates were analysed in one analytical batch. The concentration of drugs after each storage period was related to the initial concentration as determined for the samples.

2.7 Assay of Metformin HCl and Propranolol HCl in Marketed Formulation

Metformin HCl and Propranolol HCl content in the marketed formulations was estimated by current developed HPLC method. Weighed and finely powdered not less than 10 tablets. Weighed the powder accurately equivalent to about 100 mg of metformin HCl and 80 mg of propranolol HCl separately and transferred individually to 1000 mL volumetric flasks. Powder was allowed to dissolve in 3D water and the volume made up to the mark with 3D water. 1 mL of the above solution was further diluted up to 50 mL with 3D water. An aliquot of 0.1 mL of the above concentrations were added to the 0.3 mL of human plasma, 0.1 ml of internal standard solution (2 µg/ml) and 0.5 mL of 50:50 mixture of acetonitrile: methanol solution were added. Then assayed for the content of metformin HCl and propranolol HCl as per the procedure mentioned previously.

3. RESULTS AND DISCUSSION

3.1 Method Development

A RP-HPLC method in human plasma was developed for the simultaneous estimation of metformin HCl and propranolol HCl in bulk mixture and in dosage form. The chromatographic conditions were optimized to provide a better performance of the assay. The sample and standard solutions were prepared and chromatograms were recorded.

Due to the structural similarity between metformin HCl to propranolol HCl, it was chosen as the second drug to normalize erratic recoveries and to improve the precision of the analysis [19]. Hence, metformin HCl was selected as an internal standard for propranolol HCL and vice versa.

All over the plasma extraction methods, protein precipitation method with 50:50 mixture of acetonitrile: methanol was found to be optimal, which produced a clean chromatogram for a blank human plasma sample and yielded the highest and stable recovery for the analyte from the plasma. The chromatographic conditions, especially the 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 potassium dihydrogenortho phosphate buffer (40:60) as mobile phase at a flow rate of 1 ml/min and the pressure maintained at140-150 kg/cm^{2}was optimized as chromatographic conditions.

3.2 Specificity

A good separation of metformin HCl and propranolol HCl was achieved with retention times of 9.084 min and 6.132 min respectively without any interference of endogenous compounds in the plasma. Each sample was injected three times and same retention times were observed in all the cases.

3.3 Linearity

A representative calibration curves of the metformin HCl and propranolol HCl peak area over the concentration range 50-2000 ng/mL resulted in the following linear least squares regression equation: y=50.81x –260.52 and y=6.3321x+54.547 respectively, where x is the concentration of drug and y is the peak area. A good linear relationship was observed as indicated by correlation coefficient value of (r) 0.9989 and 0.9996 for metformin HCl and propranolol HCl respectively. The peak areas were reproducible as indicated by low coefficient of variation.

3.4 System Suitability

The system suitability was studied by conducting the experiment on standard solutions and observed the changes in separation, retention times, and asymmetry of the peaks. The resolution, areas, retention time, theoretical plate's values and peak asymmetry were calculated for standard and sample solutions. Results obtained are given in Table 1.

Table 1. System suitability parameters

Standard	Average	% RSD
Metformin HCl		
Retention time	9.084	0.14
Theoretical plates	1472	
Resolution	8.7	
Asymmetry	1.28	
Propranolol HCl		
Retention time	6.132	0.21
Theoretical plates	1255	
Resolution	8.1	
Asymmetry	0.94	

3.5. Accuracy and Precision

Table 2 presents the inter-day and intra-day estimation of metformin HCl & propranolol HCl of the developed HPLC method. Results indicated that, the HPLC method was highly precise which was confirmed by the low coefficient of variation (less than 1.56%) and the results are mentioned in Table 2.

% RE values were found to be within the limits of ICH guidelines indicating the high accuracy of the present HPLC method as given in Table 2. The results indicated that the method is accurate and it can be used for the estimation of metformin HCl and propranolol HCl.

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

Concentration(ng/mL)			% CV		% RE	
Actual	Measured(mean, n=5)					
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Metformin HCl						
100	99.65	99.23	1.21	1.16	-0.35	-0.77
200	199.09	200.12	1.34	1.09	-0.45	0.06
500	500.04	499.34	1.42	1.27	0.08	-0.13
1000	999.95	1000.09	1.38	1.34	-0.05	0.09
Propranolol HCl						
100	99.12	99.45	1.45	1.42	-0.88	-0.55
200	198.98	199.02	1.32	1.39	-0.51	-0.49
500	499.58	499.35	1.02	1.22	-0.08	-0.13
1000	995.75	996.58	1.56	1.40	-0.42	-0.34

3.6 Recovery

The percentage recovery of the analytical method for metformin HCl and propranolol HCl in human plasma is shown in Table 3. The results indicate that the recoveries are well within in the acceptance range. The recovery results demonstrated good efficiency of the precipitation procedure employed in this study, i.e., more than 99% of metformin HCl & propranolol HCl was detected.

Table 3. Recovery of metformin HCl and propranolol HCl from plasma drug solution

Concentration (ng/mL)	Mean percent of recovery(± s.d) (n=5)	
	Metformin HCl	Propranolol HCl
100	99.59±0.99	99.45±0.42
200	99.97±0.94	99.89±0.36
500	98.99±0.91	99.25±0.87
1000	100.03±0.87	99.65±0.21

3.7 Limit of Detection and Limit of Quantification

LOD of metformin HCl and propranolol HCl was found to be 15 ng/ml and 30 ng/ml respectively. The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of metformin HCl and propranolol HCl was found to be 45ng/ml and 50 ng/ml respectively.

3.8 Robustness

Robustness of the present analytical method was determined by altering chromatographic conditions, such as flow rate and mobile phase. Results indicated that, there were no marked changes in the chromatograms, which demonstrated that the present analytical method is robust. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which also confirms that the method is having good system suitability. Results are mentioned in Table 4.

Table 4. Robustness studies

Parameters	Retention time % RSD	Peak area % RSD
Flow rate (0.6 ml/min)		
Metformin HCl	0.04	0.24
Propranolol HCl	0.02	0.15
Flow rate (0.8 ml/min)		
Metformin HCl	0.05	0.31
Propranolol HCl	0.02	0.21
Mobile Phase (30:70)		
Metformin HCl	0.06	0.27
Propranolol HCl	0.08	0.31
Mobile Phase (50:50)		
Metformin HCl	0.07	0.24
Propranolol HCl	0.03	0.28

3.9 Ruggedness

The ruggedness of the present HPLC method was determined by carrying out the analysis on different days with different analysts and the results are mentioned in Table 5. Results shown that, the % RSD values of peak area and retention times of both drugs have been within the limits, which confirms that the developed method is rugged.

Table 5. Ruggedness studies

Parameters	Retention time % RSD	Peak area % RSD
Day 1- Analyst 1		
Metformin HCl	0.03	0.21
Propranolol HCl	0.04	0.23
Day 2- Analyst 2		
Metformin HCl	0.05	0.26
Propranolol HCl	0.05	0.31
Day 3- Analyst 3		
Metformin HCl	0.03	0.24
Propranolol HCl	0.05	0.27

3.10 Stability

The stability results of the experimental samples were mentioned in Table 6. Three freeze-thaw cycles and ambient temperature storage of the freeze quality control samples up to 12 hr prior to sample preparation appeared to have no effect on the quantitation of analyte. 12 hr room temperature and freeze-thaw cycles for low, medium and high quality control samples indicated that the drugs were stable in human plasma under experimental conditions. Auto sampler and 30-days stability results indicated that, metformin HCl and propranolol HCl samples were stable at specific conditions. Hence, conclude that the present developed HPLC method is more stable at extreme conditions.

Table 6. Stability of samples

Concentration (ng/mL)	Observed concentration(± s.d) (n=5)	
	Metformin HCl	**Propranolol HCl**
Short term stability for 12 h		
50 (Low)	99.63±0.91	98.12±0.28
100 (Medium)	99.35±0.85	99.45±0.78
2000 (High)	99.12±0.67	99.02±0.45
Three freeze and thaw cycles		
50 (Low)	99.89±0.33	99.65±0.27
100 (Medium)	99.45±0.23	99.21±0.66
2000 (High)	99.74±0.45	99.09±0.87
Auto sampler stability for 24 h (5±1ºC)		
50 (Low)	98.94±0.91	99.14±0.22
100 (Medium)	99.02±0.44	99.20±0.65
2000 (High)	99.25±0.31	99.68±0.45
30- days stability at - 70±5 ºC		
50 (Low)	99.12±0.41	98.63±0.81
100 (Medium)	99.24±0.39	99.04±0.75
2000 (High)	99.02±0.84	99.97±0.71

3.11 Assay of Metformin HCl and Propranolol HCl in Marketed Formulations

Assay of the metformin HCl and propranolol HCl in the marketed sample was estimated by the present HPLC method and the results are mentioned in Table 7. Results showed that the content of the drug in the marketed formulation was found to be more than 97%, which confirm that the developed HPLC method is more accurate.

Table 7. Analysis of marketed formulation (n=5)

Marketed formulation	Amount of drug (mg/tab)		% Assay	% RSD
	Labeled (mg)	**Estimated (mg)**		
Glycomet 500 SR	500	492.01±2	98.41 ±0.45	0.05
Ciplar LA 80	80	78.92±1.98	98.65±0.78	0.02

The extra peaks were detected in the formulation chromatogram, which might be due to excipients/polymers present in the formulation. These peaks do not interfere with the standard peaks, which clearly indorse the assay method was found to be highly specific.

4. CONCLUSION

A convenient and rapid RP-HPLC method has been developed for the estimation of metformin and propranolol HCl in bulk mixture as well as in dosage form. The developed HPLC method is standard, simple, rapid, selective, accurate, precise, rugged, robust, highly sensitive and stable. The proposed method involves no tedious extraction procedures and do not involve laborious time consuming sample preparation. It is concluded that, the present HPLC method is suitable for the routine clinical monitoring of plasma levels in human subjects for bioequivalence studies and for use in the pharmacokinetic research studies, using small laboratory animals where small aliquots of human plasma would permit the quantitation of metformin HCl as well as propranolol HCl.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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