RP- HPLC Method for Estimation of Furosemide in Rabbit Plasma

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ABSTRACT

The present RP-HPLC method for in vivo estimation of furosemide in male rabbits was developed to improve its efficiency. The method was developed as per ICH guidelines. ODS column (C_{18} 250 × 4.6 mm, 5 µm) with flow rate 1 mL/min was used and the column temperature was set at 25°C. The runtime was set for 10 min and the detection was done at λ_{max} 210 nm. The mobile phase used was phosphate buffer (pH 4.6) and acetonitrile in 30:70 ratio. The developed method had shown high specificity for furosemide in rabbit plasma and the regression was found to be R^2=0.9999 in the range of 0.1 to 5 µg/0.5 mL. The regression equation was found to be Y=0.4818X+0.0039. The lower limit of detection was found to be 0.1 µg/0.5 mL of plasma. The developed method for furosemide in rabbit plasma had shown good accuracy without any intra-day and inter-day variation. The proposed method was found to be adequate and reproducible for the estimation of furosemide in rabbit plasma.

Keywords: Furosemide; Rabbit plasma; RP-HPLC; ICH guidelines

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INTRODUCTION

Furosemide is a (5-(amino sulfonyl)-4-chloro-2-[(2-furanylmethyl) amino] benzoic acid) a potent diuretic used in the treatment of hypertension (Figure 1) [1]. The main mechanism of action of furosemide is by blocking the absorption of sodium and chloride from loop of henle. Furosemide inhibits primarily the absorption of sodium and chloride not only in the proximal and distal tubules but also in the loop of henle. The high degree of efficacy is largely due to the unique site of action. The action on the distal tubule is independent of any inhibitory effect on carbonic anhydrase and aldosterone. It is mainly used in the case of cardiac and pulmonary disorders in children and neonates. In adults the dose of furosemide is 20-80 mg and in children 1 mg/kg up to a maximum of 40 mg [2,3].
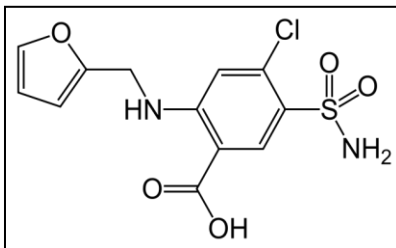

Figure 1: Structure of furosemide

Several analytical methods have been reported for the estimation of furosemide in plasma including RP-HPLC [4,5], gas chromatography [6] and dispersive liquid-liquid micro extraction spectro-fluorimetric method [7]. The gas chromatography method may require derivatisation and the process is considered tedious. The HPLC methods are simple, precise and highly accurate and require small amount of sample. However the HPLC methods using the most commonly available columns and consuming less quantity of solvents were preferred. In the present study author reports a rapid, sensitive, accurate and precise

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RP-HPLC method for the estimation of drug in the plasma of male rabbits. The study describes method for the determination of furosemide in male rabbits by using ODS C_{18} (250 x 4.6 mm, 5 µm) column.

MATERIALS AND METHODS

Materials
Furosemide was gift sample received from IPCA Laboratories, Mumbai, India. Diclofenac was gift samples received from Hetero Drugs Pvt. Ltd, Hyderabad, India. HPLC grade acetonitrile, HPLC grade methanol and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study. All the reagents and chemicals used are of analytical grade.

Instrumentation
Chromatography was performed with waters 2695 HPLC provided with high speed auto sampler, column oven, degasser and 2996 PDA detector and isocratic pump mode was used. Class Empower-2 software was used to monitor all the operations in chromatography system. ODS column (C_{18} 250 × 4.6 mm, 5 µm) with flow rate 1 mL/min was used and the column temperature was set at 25°C. The runtime was set for 10 min and the detection was done at λ_{max} 210 nm. The volume of injection loop was 20 µL and the solubility of the compounds was enhanced by sonication. All the weighing in the experiment was done on Afcoset electronic balance. The Hermlemicrolitre centrifuge Z100 (model no 292 P01) was used for the centrifugation process and Remi equipments (model no-CM101DX) cyclomixer was used.

Preparation of Phosphate Buffer (pH 4.6)
The buffer solution was prepared by dissolving 7.0 g of potassium dihydrogen phosphate in 900 mL of HPLC grade water in a 1000 mL clean and dry flask and mixed well. The volume was made to 100 mL by adding HPLC grade water by adjusting pH to 4.6 with 0.1% orthophosphoric acid and triethanolamine solution.

Preparation of Mobile Phase
Mobile phase was prepared by mixing 300 mL of phosphate buffer (pH 4.6) and 700 mL of acetonitrile in 1000 mL volumetric flask. The mixture was degassed in an ultra sonicator for 5 min and the resultant mobile phase was filtered through 0.45 µ membrane filter under vacuum. The same mobile phase was used as diluent for drug solutions in the study.

Preparation of Standard Solution
10 mg of furosemide was weighed accurately and dissolved in 7 mL of diluent in a 100 mL clean and dry volumetric flask. The solution was then sonicated for complete dissolution of drug and the final volume was made with the same solvent.
From the above prepared solution 1 mL was transferred into a 10 mL clean and dry volumetric flask and it was diluted up to the mark with the same diluent (1 µg/mL). The standard solutions prepared were injected five times and the eluents were monitored at λ_{max} 210 nm using PDA detector.

Collection of Blood Samples from Male Rabbits
Blood samples were collected in heparinized eppendroffs tube before administration of dosage form and from left marginal ear vein of rabbit (CPCSEA No.:1677/PO/Re/S/2012/CPCSEA). Plasma was separated immediately from the blood cells by centrifugation at 8000 rpm for 10 min and stored at -20°C until further study.
Extraction of drug from plasma 250 µL of plasma and varied concentrations of furosemide were taken into a teflon lined cap centrifuging tube. Diclofenac (internal standard) 50 µL of concentration 2 µg/0.5 mL and 2 mL of acetonitrile were added to plasma simultaneously. The centrifuging tubes were then cyclo mixed for 2 min and finally centrifuged for 3 min at 3200 rpm speed. Further the supernatant liquids were collected in another Eppendrof tube and 20 µL of supernatant (organic layer) was filtered through 0.2 µm membrane filter and injected in to the analytical column. Sample was injected into the analytical column were monitored at 210 nm. The concentrations were prepared in the range of 0.1 to 5 µg/0.5 mL of plasma. The experiment was repeated for five times for all the concentrations.

Validation Development
The proposed RP-HPLC method was validated in terms of linearity, precision and accuracy [8-10].

Selectivity
An aqueous mixture of furosemide (1 µg/mL) was prepared and injected in to the column to determine the retention time of the drug. Drug free plasma was chromatographed to observe any interfering peaks at the retention time of drug. Spiked

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plasma containing 1 μg/0.5 mL furosemide concentration was chromatographed to check the effect of various endogenous components at drug retention time.

Linearity
The linearity of the proposed RP-HPLC method was determined in terms of the correlation coefficient. Calibration curve was taken in to consideration. The peak area ratio of furosemide to internal standard was recorded and the regression of the plasma concentration over its peak area ratios was calculated using the least square method of analysis. Mean of five samples was taken for linearity determination.

Precision
To check the intra-day and inter-day variations of the method spiked plasma containing 0.2, 0.5, 1 μg/0.5 mL of furosemide containing 2 μg/0.5 mL of diclofenac (internal standard) was subjected to the proposed RP-HPLC method of analysis and the results obtained were noted. Each sample was extracted from plasma as described previously. The precision of the proposed method i.e. the intra-day and inter-day variations in the plasma were calculated in terms of percent coefficient of variation. Mean of five samples was taken to determine the precision.

Accuracy
To determine the accuracy of the proposed RP-HPLC method, recovery studies were carried out by analyzing the plasma samples containing different concentrations of drug (0.2, 0.5, 1 μg of furosemide). Each sample was injected three times. The difference in the measured concentration and that of the added quantity was expressed in terms of percent recovery.

RESULTS AND DISCUSSION

Selectivity
The run time of the method was set at 10 min. The retention time obtained for furosemide and diclofenac were 4.7 minutes and 4.02 minutes respectively and was shown in model chromatogram (Figure 2). The same furosemide solution containing diclofenac as internal standard was injected for five times and the retention time was found to be same.
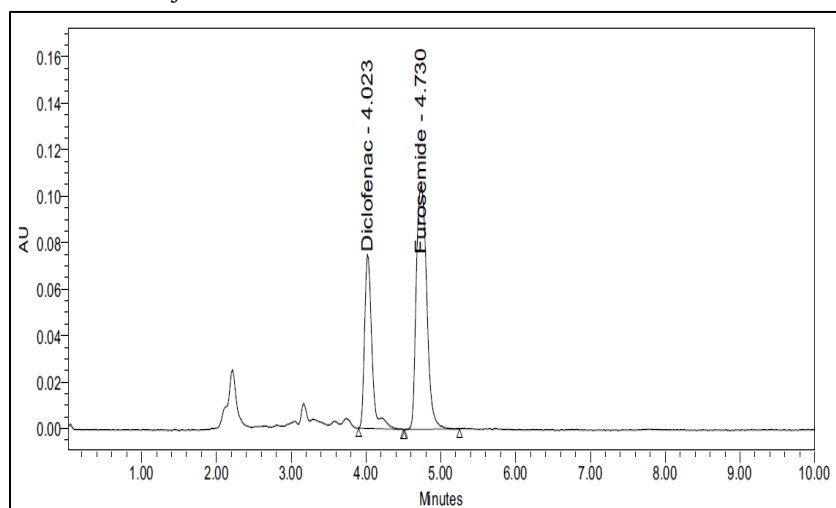

Figure 2: A model chromatogram for the HPLC estimation of furosemide in rabbit plasma

Standard Calibration Curve
The standard calibration curve of peak area ratio of furosemide to internal standard for five determinations (Figure 3) and the results were reported (Table 1). The peak area ratios of furosemide to internal standard were reproducible as indicated by low coefficient of variation (0.50).

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Table 1: Calibration of for the estimation of furosemide in rabbit plasma

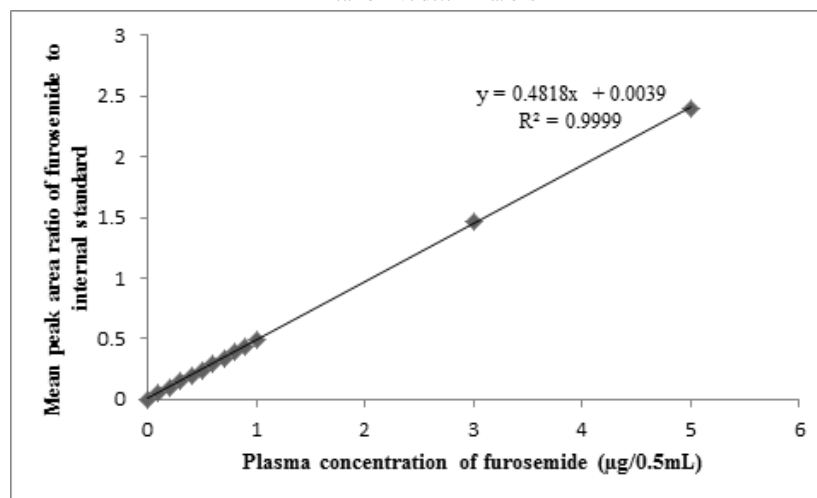| Concentration of furosemide (µg/0.5 mL)	Mean peak area ratio*	% C.V.
0	0	0
0.1	0.0489	0.5
0.2	0.1005	0.89
0.3	0.1484	1.18
0.4	0.1984	2.11
0.5	0.2421	0.59
0.6	0.2881	0.55
0.7	0.3348	0.86
0.8	0.3917	0.71
0.9	0.4387	1.32
1	0.4924	2.11
3	1.4712	1.34
5	2.3993	0.69

*Mean of five determinations

Figure 3: Standard graph for the estimation of furosemide in rabbit plasma

Linearity

The linearity range of proposed RP-HPLC method in plasma was calibrated by using standard curve by plotting peak area ratios of furosemide to internal standard against their concentrations. The regression analysis of the calibration curve was done by least square method, a high correlation coefficient was observed (R^2=0.9999) in the range of 0.1 to 5 µg/0.5 mL (Figure 3). The regression equation was found to be Y=0.4818X+0.0039 where Y is the peak area ratios of furosemide to internal standard and X is the concentration of furosemide. The lower limit of detection is 0.1 µg/0.5 mL.

Precision

The precision of the proposed RP-HPLC method i.e. the intra-day and inter-day variations in the peak areas of drug solutions in plasma were calculated in terms of percent coefficient of variation and the results were reported (Table 2). Different concentrations of drug in plasma were prepared and the filtrate samples were repeatedly injected on the same day and on three different days for five times. The coefficient of variation in the peak area of furosemide for five replicate injections was found to be less than 2.75%. Also the inter-day variation was found to be less than 2%. Thus the results show that the present HPLC method is highly reproducible.

Table 2: Inter-day and intra-day precision of the HPLC method for the estimation of furosemide in rabbit plasma

Plasma concentration of furosemide (µg/0.5mL)	Concentration of furosemide (µg/0.5mL) found			Concentration of furosemide (µg/0.5mL) found		
	(Intra-day)			(Inter-day)		
	Mean*	%CV	%Precision	Mean*	%CV	%Precision
0.2	0.196	2.75	99.2 ± 2.75	0.195	2	97.5 ± 2
0.5	0.497	1.38	99.8 ± 1.38	0.495	1.45	99 ± 1.45
1	0.999	1.24	99.9 ± 1.24	0.995	0.99	99.5 ± 0.99

*Mean of five determinations

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Accuracy

The difference between the measures concentration and the added concentration of the drug was expressed in terms of recovery. The percent recovery was found to be between $99.5 \pm 0.19\%$ - 97.5 ± 1.25 and the reproducibility was found to be satisfactory. The results were reported (Table 3).

Table 3: Recovery of furosemide after adding known amounts to pre-analyzed plasma samples (1µg/0.5mL)

Amount of furosemide added (µg)	*Mean±SD amount (µg) recovered	% Recovery
0.2	0.195 ± 0.03	97.5 ± 1.25
0.5	0.498 ± 0.14	99.6 ± 1.42
1	0.995 ± 0.19	99.5 ± 1.09

*Mean of three determinations

CONCLUSION

The present RP-HPLC method was validated as per ICH guidelines i.e., by linearity, accuracy and precision. The method was found to be simple, precise, highly accurate and reproducible for the estimation of furosemide in rabbit plasma.

ACKNOWLEDGEMENTS

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