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A Validated High Performance Liquid Chromatographic Method for the Estimation of Furosemide in Spiked Human Plasma

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ABSTRACT

A modified liquid- liquid extraction based reverse phase high performance liquid chromatographic method has been developed and validated for the determination of Furosemide in human plasma. The chromatographic separation was achieved with Pinnacle C18 column (250 x 4.60 mm, 5µm i.d.) and a mobile phase comprising of a mixture of Methanol: Water: Triethylamine (70:30:0.1v/v/v), pH adjusted to 3.2 with orthophosphoric acid was used with UV detection at 274nm. The internal standard spironolactone structurally related to Furosemide was used. The retention time for Furosemide and spironolactone was found to be 3.75min and 8.12min respectively. A calibration curve was linear in the concentration range of 200-2200ng/ml for Furosemide in blank human plasma. The % recovery from human plasma was found to be in the range of 89.41 to 93.91 for Furosemide. The lower limit of quantitaion was found to be 200ng/ml and no interference was found from endogenous compound. The specificity, linearity, accuracy, precision and stability of the method were evaluated from spiked human plasma sample as per EMA (European Medicine Agency) guideline. The method can be used for supporting therapeutic drug monitoring and pharmacokinetic studies.

Keywords: Furosemide, Spironolactone, High Performance Liquid Chromatography, Human plasma.

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INTRODUCTION

Furosemide, 5-(aminosulfonyl)-4-chloro-2-[(2 furanylmethyl) amino] benzoic acid (Figure 1), a diuretic drug[1] has been used in the treatment of congestive heart failure and edema. Furosemide acts on thick ascending limb of loop of Henle and block the $K^+/Na^+/2Cl^-$ co-transporter leading to decrease in sodium and chloride reabsorption and increasing the excretion of potassium in the distal renal tubule[2]. A number of high performance liquid chromatographic methods have been reported for furosemide[3-11]. HPLC-mass spectrometric analysis[12] have been used for measurement of Furosemide in biological fluids and urine. In this research article, we have developed a modified method for estimation of Furosemide in blank human plasma with simple liquid liquid extraction method. The advantages of proposed method over reported method are; (1) Methanol: Water: Triethylamine (70:30:0.1v/v/v), pH adjusted to 3.2 with orthophosphoric acid, mobile phase optimized which was economical and having shorter run time of 1ml/min. (2) No Interference of endogenous substances from plasma with std drug. Retention time of blank plasma and standard drug was found 2.1 and 3.75min respectively. (3) Simple liquid liquid extraction method was used for extraction of standard drug from blank plasma, save considerable time (4) Evaluation of stability was carried out, which ensured that storage conditions used did not affect the concentrations of the analyte. (5) The use of internal standard spironolactone structurally related to Furosemide minimizes error during sample preparation. In conclusion the modified method developed for estimation of Furosemide in blank human plasma was simple, selective, precise, accurate, and cost effective. The resolution factor found between blank plasma and standard drug was more than 1.5.

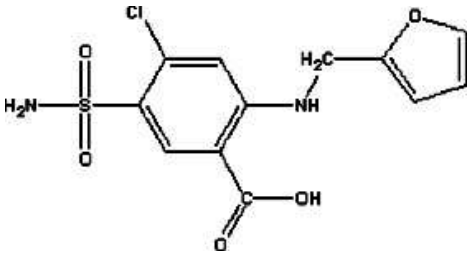

Figure 1: Chemical structure of Furosemide

MATERIALS AND METHOD

Chemicals and Reagents

Standard drug Furosemide and internal standard Spironolactone were obtained as gift sample from Aventis Pharma Ltd. Methanol, Water, Triethylamine were of HPLC grade and Orthophosphoric acid AR grade, purchased from Thermosil Chemicals Ltd. (Pune. India). The controlled human

plasma was procured from Blood Blank (Pune). Whatman filter paper no. 41(Millipore, USA) were used in the study.

Instrumentation and Chromatographic Conditions

Jasco HPLC system consisting of Jasco PU2080 HPLC pump and UV2075, UV/VIS detector and JASCO Borwin 1.50.8.0 version software was used for analysis. Separation was carried out on Pinnacle C18 (250 x 4.60 mm, 5µm i.d.) using mobile phase consisted of Methanol: Water: Triethylamine (70:30:0.1v/v/v), pH adjusted to 3.2 with orthophosphoric acid, it was filtered through 0.45µ membrane filter paper using membrane filtration assembly and then sonicated on ultrasonic water bath for 10 min. The Gaussian peak shape with perfect symmetry was obtained at retention time of 3.75min. Drugs which have same solubility and considerable absorbance at 274nm of standard Furosemide were tried as internal standards, based on that spironolactone was selected as internal standard (100µg/ml). Spironolactone was well extracted from the liquid-liquid extraction technique that was developed for Furosemide. The acceptable system suitability parameter obtained summarized in Table 1.

Table 1: System suitability parameter

Parameters	Furosemide	Spironolactone(IS)
Concentration	1µg/ml	100µg/ml
Retention Time (min.)	3.75	8.12
Tailing Factor	0.8	1.01
Theoretical plate	4263	3498
Area	86340	97104

Preparation of standard stock solution of Furosemide

Standard stock solution of Furosemide was prepared by dissolving 10 mg of Furosemide in 10 ml methanol to get concentration 1000µg/ml. 0.1ml of stock solution diluted separately to 10ml with mobile phase in another volumetric flask to get working standard solution of 10µg/ml concentration.

Preparation of stock solution of internal standard (IS)

10mg of Spironolactone (IS) was dissolved in 10ml methanol to get concentration 1000µg/ml. 1ml of stock solution diluted separately to 10ml with mobile phase in another volumetric flask to get working standard solution of 100µg/ml concentration.

Preparation of blank plasma sample solution

1.6ml of blank plasma sample was pipette out in glass tube and 4ml methanol was added, vortexed for 10mins then centrifuged for 10mins at 3000 rpm. Decant the supernatant and organic phase

was transferred to a second clean tube. The separated organic phase was evaporated. To the dried residue 0.5ml of mobile phase was added and which was directly injected to HPLC system.

Preparation of spiked plasma sample solution

To 1.6ml blank plasma, 0.2ml Spironolactone (IS) and Furosemide working standard (concentration of solution 2, 6, 10, 12, 18, 22µg/ml) was added to get 200, 600, 1000 1200, 1800 and 2200ng/ml of Furosemide and 100µg/ml of Spironolactone spiked plasma concentration. For extraction of Furosemide and Spironolactone from spiked plasma sample methanol was used as extracting solvent. 1ml of spiked plasma sample of different concentration was pipette out in different glass tube and 4ml methanol was added, vortexed for 10mins then centrifuged for 10mins at 3000 rpm. Decant the supernatant and organic phase was transferred to a second clean tube. The process of extraction was repeated three times. The separated organic phase was evaporated. To the dried residue 0.5ml of mobile phase was added and which was directly injected to HPLC.

Selection of analytical wavelength

10 mg of Furosemide was dissolved in methanol to make up volume to 10ml in volumetric flask to get Furosemide standard stock solution of concentration 1000µg/ml. 0.1 ml of standard stock solution of Furosemide and spironolactone (1000µg/ml) diluted with mobile phase to get working standard solution of 10µg/ml concentration and scanned over the range of 200-400 nm. It was observed that standard drug and IS shown considerable absorbance at 274 nm.(Figure 3)

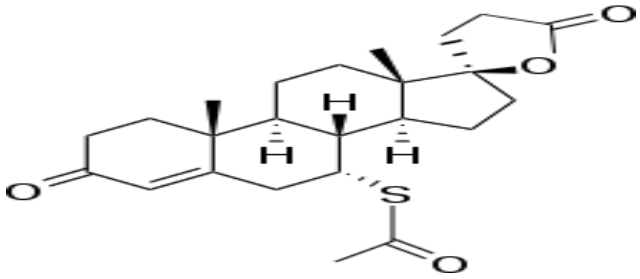

Figure 2: Chemical structure of Spironolactone

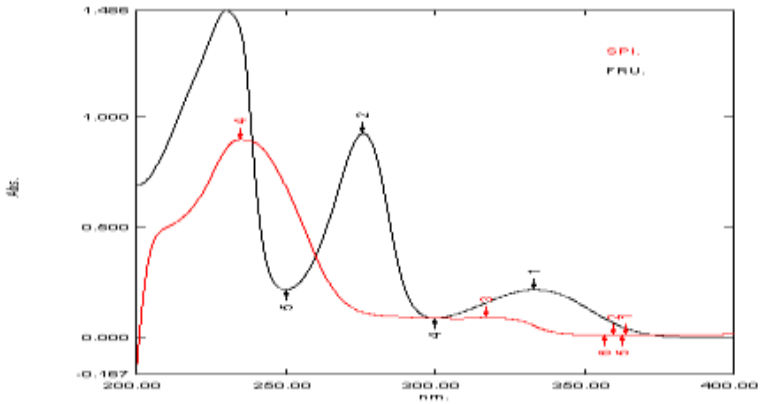

Figure 3: Overlain UV spectra of standard Furosemide and Spironolactone (IS) (10µg/ml)

Method Validation

The developed method was validated as per EMA (European Medicine Agency) guideline[13]. The selectivity of the method was evaluated by repeated analysis of human plasma sample spiked at lower limit of quantification (LLOQ-200ng/ml). The precision and accuracy at LLOQ-200ng/ml found within 15.00% which meet the acceptance criteria. Linearity was tested for Furosemide standard and blank plasma spiked with Furosemide in the concentration range of 200-2200ng/ml and internal standard spironolactone (100µg/ml). The five replicates per concentration were injected into HPLC system and peak areas were calculated. The Peak areas were plotted against the corresponding concentrations to obtain the calibration curve. The data were best fitted by linear equation Y = mx + c. The accuracy expressed as percentage recoveries. The % recoveries were determined by measuring the responses of the extracted plasma quality control samples against standard quality control samples using five determinations of four different concentrations covering the calibration curve range; The LLOQ 200ng/ml, LQC 600ng/ml, MQC 1200ng/ml, HQC 1800ng/ml as per EMEA guideline. Precision is expressed as the coefficient of variation (CV). Within run accuracy was determined using five determinations of four different concentrations at LLOQ, LQC, MQC, and HQC level on the same day. The accuracy and Precision of the method was evaluated for different concentration at LLOQ, LQC, MQC, HQC level (200, 600, 1200, 1800ng/ml). Between run accuracy was determined using five determinations of four different concentrations at LLOQ, LQC, MQC, HQC level. Stability studies of spiked quality control samples for LQC 600ng/ml and HQC 1800ng/ml levels were determined. Freeze thaw stability of the spiked quality control samples was determined after three freeze thaw cycles stored at -28^{o}C. Short term stability of the spiked quality control samples was determined for a period of 5 hours stored at room temperature. Long term stability of the spiked quality control samples was determined for a period of 1 month stored at in refrigerator. Stability of standard drug stock solution (100µg/ml) and working solutions of the analyte (LQC and HQC) and internal standard spironolactone (100µg/ml) was determined for a period of 5 days stored in refrigerator. All stability samples compared against freshly prepared quality control samples and assessed for stability.

RESULTS AND DISCUSSION

Linearity was tested for Furosemide standard and blank plasma spiked with Furosemide in the concentration range of 200-2200ng/ml. The correlation coefficients were found to be 0.999 for Furosemide standard and blank plasma spiked with Furosemide (Figure 4 & 5). The chromatogram

of blank plasma(Figure 6) and plasma spiked with furosemide and internal standard spironolactone (Figure 7) shown that no endogenous interference noted at the retention time of the drugs as well as analytical method was able to differentiate the analyte of interest and IS from endogenous components in the matrix indicated stability of method. The retention time of standard furosemide and spironolactone were found 3.75 and 8.12 min. The accuracy and Precision of the method were evaluated for different concentration at LLOQ, LQC, MQC, HQC level (200, 600, 1200, 1800ng/ml). The results are given in Table 2 and 3. The % CV found for within run precision and between run precision was 0.79 to 2.80 % and 0.68 to 2.80 % respectively. The % Recovery was found in the range of 89.41 to 93.91% which is within acceptance limit 85-115.00%. The results indicated that method was precise and accurate. The spiked quality control samples of drug Furosemide was stable found to be stable after three freeze thaw cycles stored at -28^{o}C,short term stability of the spiked quality control samples for a period of 5 hours stored at room temperature, long term stability of the spiked quality control samples stored in refrigerator for 1 month. Standard drug stock solution (100µg/ml) and working solutions of the analyte (LQC and HQC) and internal standard spironolactone (100µg/ml) was found to be stable for a period of 5 days stored in refrigerator. The results obtained are given in Table 4.

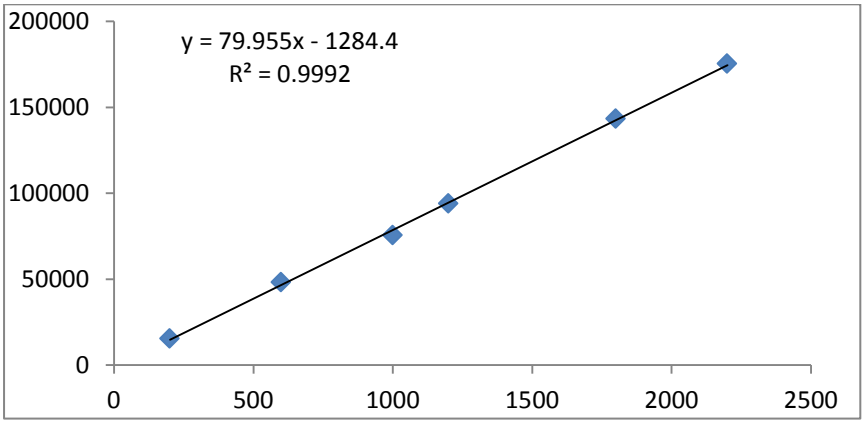

Figure 4: Linearity graph of Standard Furosemide 200-2200ng/ml

Table 2: Results for Within Run Precision and Accuracy of Spiked Furosemide Sample (n= 5)

Parameters Evaluated	LLOQ 200ng/ml		LQC 600ng/ml		MQC 1200ng/ml		HQC 1800ng/ml	
	Std	Spiked Plasma	Std	Spiked Plasma	Std	Spiked Plasma	Std	Spiked Plasma
Mean Peak Area	15888	14700.8	48157.7	43060	93600.5	85397	143364	128348
SD	401.455	411.534	872.321	570.122	839.153	775.106	1126.06	1629.33
%CV	2.52678	2.79941	1.81139	1.32402	0.89653	0.90765	0.78545	1.26947
%Recovery	92.52%		89.41%		91.23%		89.52%	

Acceptance Criteria:

% CV at each QC level should be ≤ 15%.

% mean recovery at each QC level should be within 85.00 to 115.00%

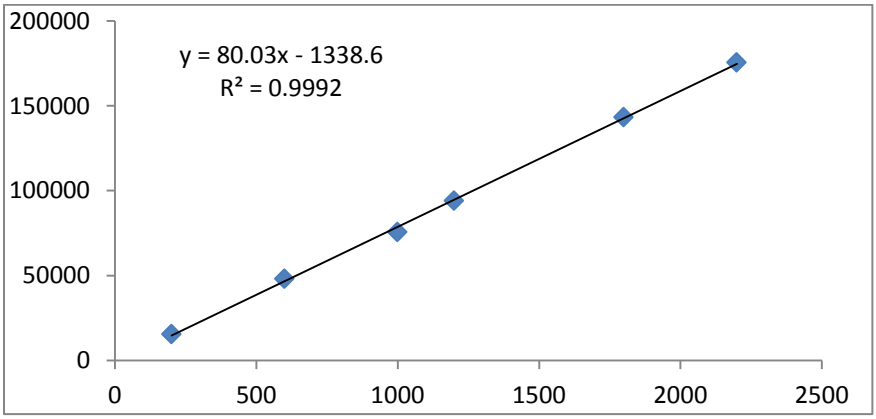

Figure 5: Linearity graph of Furosemide spiked plasma. 200- 2200ng/ml.

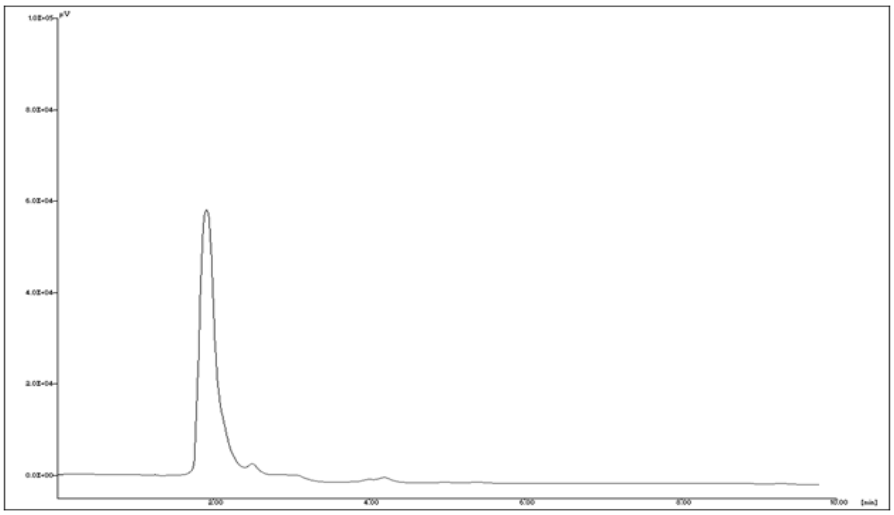

Figure 6: Chromatogram of blank human Plasma

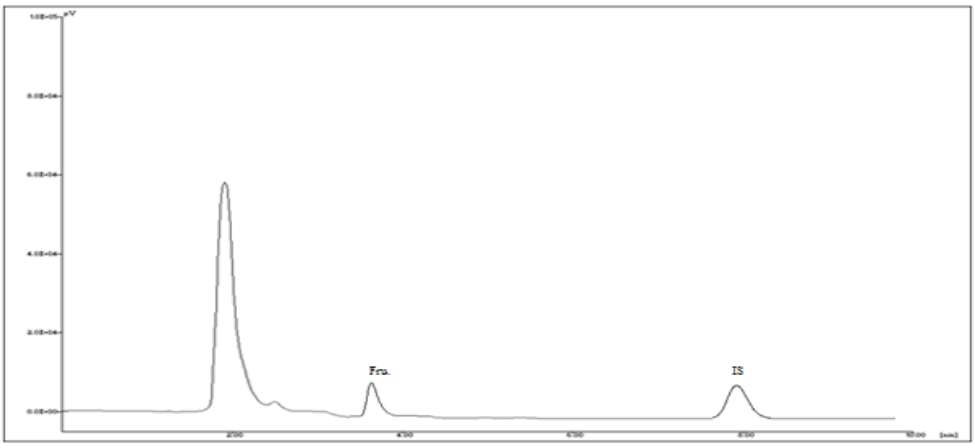

Figure 7: Chromatogram of blank human plasma spiked with Std. Furosemide 1µg/ml and internal standard spironolactone 100µg/ml

Table 3: Results for Between Run Precision and Accuracy of Spiked Furosemide Sample

Nominal concentration ng/ml	Mean amount of drug found ng/ml			Mean from three days ± SD	%Recovery	%CV
	Day-I	Day-II	Day-III			
200	185.05	191.62	186.83	187.82± 3.38	93.91	1.79
600	536.49	553.53	543.83	544.62± 6.85	90.77	1.25
1200	1094.83	1106.32	1100.22	1100.46± 4.31	91.70	0.39
1800	1611.46	1659.40	1649.08	1639.98± 7.21	91.11	0.44

Acceptance Criteria:

% CV at each QC level should be ≤ 15%.

% mean recovery at each QC level should be within 85.00 to 115.00%.

Table 4: Stability study data for Furosemide in plasma

Stability Study Data	Nominal concentration g/ml			
	600 ng/ml		1800 ng/ml	
	% Mean Stability	%CV	% Mean Recovery	%CV
freeze thaw cycles	98.27	1.93	100.94	1.26
short term stability	97.67	1.38	100.47	0.69
long term stability	99.14	0.95	100.93	1.26
Standard drug solution stability	99.27	1.55	100.38	0.27
Internal standard stability (100 µg/ml)	100.40	0.53	-	-

Acceptance Criteria

% CV at each QC level should be ≤ 15%

% mean stability at each QC level should be within 85.00 to 115.00%.

CONCLUSION

In RP- HPLC method both standard Furosemide and internal standard spironolactone were successfully resolved using methanol and water in ratio of 70:30v/v and pH adjusted to 3.2 using triethylamine AR grade, orthophosphoric acid AR grade, at flow rate-1ml/min. As compared to reported method in the developed RP-HPLC method, no endogenous interferences were noted at the retention time of the drugs. The drugs were satisfactorily resolved from plasma peaks with tR values 3.75 min for frusemide and 8.12 min for internal standard spironolactone. The developed method was found economical precise accurate and stability study data indicated that ensured that storage conditions used did not affect the concentrations of the analyte.

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