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Estimation of Amiloride and Furosemide in human plasma using high performance liquid chromatography

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ABSTRACT

A novel, simple, sensitive and precise RP-HPLC method has been developed and validated for quantitative analysis of amiloride and furosemide in human plasma. Use of a Inertsil (250 mm × 4.6 mm) 5-µm particle, C18 column with methanol: 0.1%glacial acetic acid (43:57, v/v) of pH 5.05 adjusted with sodium hydroxide as isocratic mobile phase at a flow rate of 1.0 mL/min. The reconstituted method involved the protein precipitation with methanol, followed by centrifugation and clear supernatant solution injected in to the column enabled separation of the drug from plasma. The detection was carried out using a UV-PDA detector at 226 nm. The method was validated for linearity, accuracy (recovery), precision, specificity and robustness. The linearity of the method was excellent over the range 100-350 ng/mL (correlation coefficient 0.999) for amiloride and 800-2800 ng/mL (correlation coefficient 0.997) for furosemide. The retention time for amiloride and furosemide was found to be 4.36 ± 0.3 and 10.2 ± 0.3 min respectively. The limit of detection was established as 50 ng/mL for amiloride and 100 ng/mL for furosemide. The accuracy was observed 97.75-101.2 % for amiloride and for furosemide 98.93%-100.2%. The recovery observed for amiloride of low, middle and high quality control samples was found to be 73.51 %, 75.19 % , 80.01 % respectively and for furosemide was found to be 71.9 %, 79.5 % and 86.4 % respectively. The stability of amiloride and furosemide was excellent, with no evidence of degradation during sample proceeding.

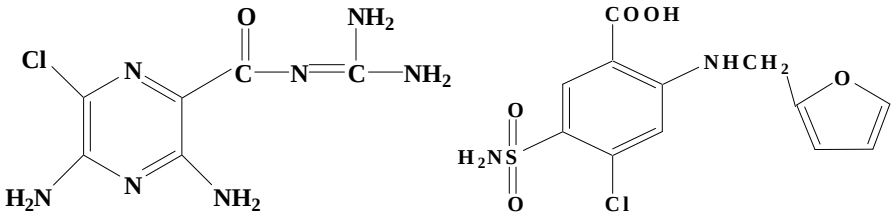
Keywords: Amiloride, Furosemide, Human Plasma, RP-HPLC, LQC, HQC.

INTRODUCTION

Amiloride (N-amidino-3, 5-diamino- 6-chloropyrazine-2-carboxamide, AML), is a photosensitive yellow or yellowish-green and odorless powder, sparingly soluble in MeOH

and slightly soluble in water, imparting acidic character to its solutions. The drug, available as the dihydrate, behaves as a mild diuretic and acts blocking the Na^+ channels in the late distal tubules and collecting ducts. By increasing the loss of sodium and chloride ions while reducing the excretion of potassium [1]. Furosemide (4-chloro-*N*-furfuryl-5-sulphamoylanthranilic acid, FRS) is a white or slightly yellow powder, practically insoluble in water but sparingly soluble in methyl alcohol (MeOH) and soluble in aqueous alkaline solutions. This drug is a potent diuretic that inhibits the reabsorption of electrolytes in the ascending limb of the loop of Henle and also in the renal tubules. While FRS has no clinically significant effect on carbonic anhydrase, it enhances water excretion, increasing loss of sodium, chloride and potassium ions. The association of FRS and AML (Fig. 1) furnishes a valuable natriuretic agent with a diminished kaliuretic effect, minimizing the risk of alkalosis in the treatment of refractory oedema associated with hepatic cirrhosis or congestive hearth failure [1]. Both individual drugs are official in the USP 24 and the BP 98. Since amiloride and furosemide make up various diuretics which are administered worldwide to humans, it is necessary to develop a simultaneous determination of these compounds in different matrixes.

Furosemide has been individually determined in pharmaceutical formulations by extractive-spectrophotometry [2], also, in biological fluids and urine it has been determined by HPLC [3-6] and HPLC-mass spectrometric analysis [7]. Amiloride on the other hand, has been individually determined in biological fluids like urine and blood-plasma, utilizing isopotential fluorimetry [8], HPLC [9], by capillary zone electrophoresis using fluorescence detection [10] and by electrochemical techniques [11]. Different methods have been presented for the determination of amiloride in presence of other drugs in pharmaceutical formulations [12-16] and in biological fluids [17]. In the case of determination of furosemide together with other drugs, their determination has been reported in tablets and urine by HPLC-EC [18], by micellar electrokinetic chromatography [19] and by HPLC [20, 21]. The simultaneous determination of amiloride and furosemide together with other drugs has been reported in urine by screening of diuretics using isocratic reversed phase LC with micellar organic mobile phase [22] and by HPLC using a micellar mobile phase of sodium dodecyl sulfate [23]. Few methods have been presented for the simultaneous determination of amiloride and furosemide. A HPTLC [24] and UV [25, 26] methods has been described for determination of both drugs in pharmaceuticals and HPLC [27] method for biological fluids. Although at present it is easy to find commercial pharmaceutical formulations containing both drugs, the analytical simultaneous determination has not been reported yet in the actual pharmacopoeia [28]. The present work was undertaken with an objective to develop a simple and sensitive direct estimation RP-HPLC method for the simultaneous determination of AML and FRS in plasma, for their pharmacokinetic studies and therapeutic drug monitoring.



Amiloride **Furosemide**

Fig. 1 Chemical structures of Amiloride and Furosemide

MATERIALS AND METHODS

Instrumentation

Liquid chromatographic system from Shimadzu comprising of manual injector, double reciprocating plunger pump LC10 ATvp for constant flow and constant pressure delivery and Photodiode array detector SPD-M10 Avp connected to software Class M10A for controlling the instrumentation as well as processing the data generated was used.

Reagents and chemicals

Amiloride (purity 100.1%) and furosemide (purity 99.8%) were obtained from Cadila Pharmaceutical Ltd. Ahemdabad, Gujarat (India). Methanol and glacial acetic acid of HPLC grade were procured by Merck Ltd., India. Sodium hydroxide was procured from Merck Ltd., India. The 0.45-µm Nylon pump filter was obtained from Advanced Micro Devices (Ambala Cantt., India). HPLC grade distilled water was used throughout the experiment. Other chemicals used were of analytical or HPLC grade. Drug free human plasma was obtained from blood bank of Bhopal Memorial and Research Centre, Bhopal, MP (India) and it was pooled from 20 fasted subjects.

Chromatographic condition

The chromatography was performed by employing an Inertsil-ODS-C18 column (250 mm × 4.60 mm, 5-µm) with UV detection at 226 nm. The isocratic mobile phase consisted of methanol: 0.1%glacial acetic acid (43:57, v/v) of pH 5.05 adjusted with sodium hydroxide. The flow rate was set at1.0 mL/min. Before use it was filtered through a 0.45-µm Nylon filter and degassed in an ultrasonic bath. The injection volume was 20µL. Peak homogeneity was expressed as peak purity and was obtained directly from the spectral analysis report obtained by use of the Class M10A software.

Method development & validation

The developed method has been intensively validated as per bioanalytical guidelines [29- 31], using validation parameters viz System suitability, linearity, LOQ, accuracy, precision, extraction recovery and freeze thaw and bench top stability. LOQ is the minimum analyte concentration that can be accurately and precisely quantify by the method.

Standard preparation

Standard stock solution of 100 µg/mL and 800 µg/mL of AML and FRS respectively were prepared separately by dissolving appropriate amounts drugs in methanol. A homogenous mixed plasma stock of 10 µg/mL and 80 µg/mL of AML and FRS were prepared by spiking 1.0 ml of respective standard stock solutions. Standard calibration solutions were prepared by further dilution of mixed plasma stock with blank plasma to get final concentrations ranging from 100-350 ng/mL and 800-2800 ng/ mL of AML and FRS respectively.

Plasma sample preparation

To 1 ml of plasma 2.5 ml of methanol was added, mixed thoroughly and vortexed for 5 min at room temperature. Solution was then centrifuged at 12000 rpm for 15 min at 10°C.The clear supernatant liquid was removed, filtered through 0.45 µ syringe filter and injected directly into HPLC system.

Specificity

To evaluate the specificity of the method, drug free plasma sample was carried through the assay procedure and retention times of the endogenous compound in the plasma were

compared with those of AML and FRS. Specificity of the method was assessed to test the matrix influence between different plasma samples.

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) of method were determined by calculating the standard deviation of the response of lowest standard on the calibration curve and the slope of calibration curve of analyte. The limit of detection and limit of quantitation were calculated by LOD = 3.3σ/S and LOQ = 10σ/S respectively, (σ = the standard deviation of the response, S = the slope of the calibration curve).

Extraction recovery

Recovery of AML and FRS was evaluated by comparing the mean peak areas of five extracted low, medium and high quality control samples with the mean peak areas of five neat reference solutions containing the same amount of the test compound.

Precision and accuracy

The intra-day, inter-day and analyst to analyst precision and accuracy of the developed method were evaluated in plasma samples spiked with AML and FRS. Intra-day, inter-day and analyst to analyst precisions were carried out at nominal concentration of 100, 200 and 300 ng/mL and 800, 1600 and 2400 ng/mL for AML and FRS respectively. The intra-day precision was evaluated five times in day, inter-day precision was evaluated on five consecutive days and analyst to analyst precision was evaluated by five different analysts.

Stability

The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times [30].

Bench top stability

Three aliquots of each of the low and high concentrations was thawed at room temperature and kept for 24 hours and analyzed at 0, 12 and 24 hrs. Concentrations were calculated to determine % change in comparison to freshly prepared samples of same concentrations.

Freeze thaw stability

Analyte stability was determined after three freeze (-70°C) and thaw cycles. Three aliquots at each of the low and high concentrations was stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 hours under the same conditions. This cycle was repeated two more times and analyzed on each cycle.

RESULTS AND DISCUSSION

Method development

During method development, number of variables was optimized to get early elution and symmetric peaks with good resolution.

Selection of precipitating agent

Organic solvents like methanol, ethanol, acetonitrile and aqueous salt solutions (Sodium sulphite, zinc sulphate solution of various strengths) were tried for precipitation of plasma proteins. Sodium sulphite solution up to 21% concentration requires very low temperature of 2°C while zinc sulphate solution requires high speed of rotation 18000 rpm. These astringent conditions are difficult to maintain during the whole experimentation, therefore methanol and

acetonitrile were tried. Acetonitrile and methanol gives complete precipitation but recovery of analytes was better with methanol i.e. 73.51% and 71.9% for AML and FRS respectively. Thus based on completeness of precipitation, drugs stability, peak characteristics and efficient recovery methanol was preferred as precipitating agent.

Wavelength selection

Amiloride exhibited two peaks at 224 and 284 nm, while furosemide shows two absorption maxima at 230 and 277 nm (Fig. 2-3). Based on the spectral characteristics and overlay spectra 226 nm was selected as the wavelength for detection. Although it is not the absorption maxima of any of the drug but at this wavelength both the drugs show considerable absorption.

Mobile phase selection

Taking into consideration of system suitability parameter like RT, Tailing factor, Number of theoretical plates, HETP and other peak response like capacity factor, peak asymmetry and resolution of drugs from plasma components, the mobile phase containing varying percentages of organic phase and buffer of different pH were tried. Initially reverse phase LC separation was tried to develop using methanol and acetate buffer pH 4.6 (60:40%) as mobile phase, in which peak of AML merged with plasma. To consider pKa value of AML (8.7) and FRS (9.9), the neutral mobile phase at pH 7 consisting of methanol and water in the ratio of 90:10 was used, FRS elutes at 14 mins as broad peak Therefore, to reduce retention time along with optimum resolution acidic mobile phase varying from pH 5.0 to 2.5 were tried. When methanol: phosphate buffer (60:40) was tried problem with AML peak shape and merging with plasma peak persists. Then phosphate buffer is replaced with 0.1% glacial acetic acid pH 5.05 which results in better resolution and sharp peaks so that methanol: 0.1% glacial acetic acid pH 5.05 (43:57%v/v) was selected as mobile phase.

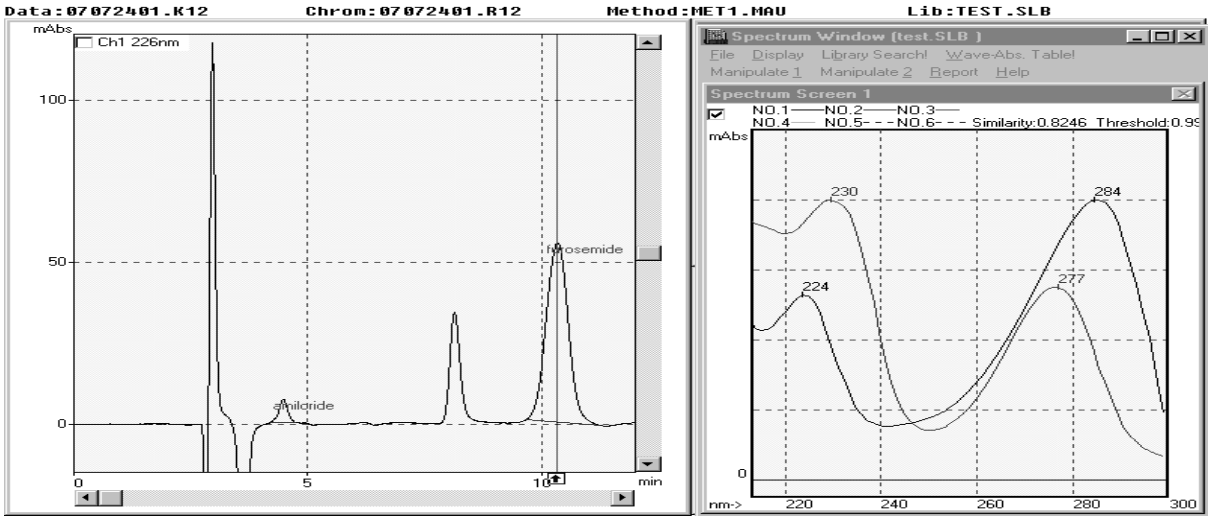


Fig.2 Representative Chromatogram of Amiloride and Furosemide (Low Quality Control)

Flow rate selection

Flow rates between1.0 and 1.5 mL/ min was tried. At a flow rate of 0.5 ml peak of furosemide was broadened with longer retention of 14 mins, while at the flow rate of 1.5 mL/ min. resolution between AML and FRS was less than 1. Flow rate of 1 mL/min gave an optimal signal-to-noise ratio with a reasonable separation time. Using a reversed-phase ODS column, the retention time was observed to be 4.36 and 10.2 for AML and FRS respectively, with a total run time of 11 min (Fig. 2-3).

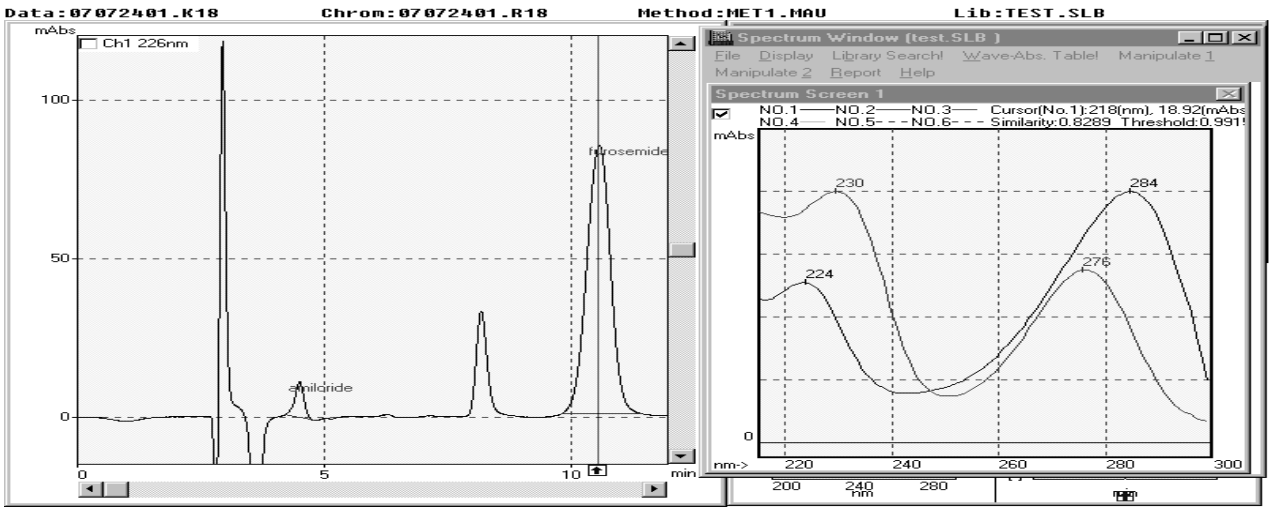


Fig. 3 Representative Chromatogram of Amiloride and Furosemide (High Quality Control)

Method Validation

System suitability

System suitability parameters were analyzed to check the system performance consistency. For system suitability parameters six replicates of MQC (Middle quality control) of both drugs in plasma were injected separately and column performances like tailing factor, height equivalent to theoretical plates (HETP), retention time (RT), area under the curve (AUC) and number of theoretical plates were observed Table-1 and % RSD values for these parameters were found far less than 15%, which indicates acceptance of system performance.

Table 1. System suitability parameters (n=6)

Parameters	AML	SD	%RSD	FRS	SD	%RSD
No. Theoretical Plates	2932.16	34.64	1.18	2252.5	26.71	1.18
Tailing factor	1.06	0.02	1.88	0.946	0.02	2.11
HETP (mm)	0.089	0.006	0.25	0.110	0.002	1.81
RT (min)	4.47	0.03	0.67	10.23	0.03	2.43

Linearity and lower limit of quantitation (LLOQ)

Linearity was assessed using six different concentrations in five replicates. The method was found to be linear in concentration range of 100-350 ng/mL and 800-2800 ng/mL for AML and FRS respectively. The linear regression equations were found to be Y (AML) = 10080 x conc. + 105.5 with (r2=0.999) and Y (FRS) = 72255 x conc. + 1030.5 (r2=0.997). The following conditions should be met in developing a calibration curve when ≤ 20% deviation of the LLOQ from nominal concentration and ≤ 15% deviation of standards other than LLOQ from nominal concentration [30]. It was obtained by serial dilution method in which high concentrations was diluted with blank plasma and quantified till concentration shows accurate and reproducible results. The LLOQ were found 100 ng/mL and 800 ng/mL for AML and FRS respectively. The LOD of the method was found to be 50 ng/mL and 100 ng/ mL for AML and FRS Respectively.

Accuracy

Accuracy was determined by analyzing three dilutions of known concentration in five replicates. The results of accuracy were expressed in terms of % nominal concentration and it

was observed for AML in between 97.75-101.2 % and for FRS in between 98.93- 100.2 % Table-2.

Precision

Repeatability

Three different levels of dilutions high quality control (HQC), medium quality control (MQC) and low quality control (LQC) samples for both drugs in five replicates were analyzed in same day for repeatability and % RSD for the both drugs were found far less than 15% (Table-2), which is acceptable limit of the developed bio-analytical method.

Intermediate Precision

Day-to-day and analyst-to-analyst variation was analyzed using three dilutions in five replicates on five different days with five analysts. Although % RSD value for FRS is higher than AML, but all the results of both drugs fall within acceptable limits Table-2.

Table 2. Results of method validation (n=5)

Drug	Nominal concentration (ng/ml)	Intra-day precision(Repeatability)			Intermediate precision					
					Inter-day precision			Analyst to analyst precision		
		Found concentration (ng/ml)	% RSD	% Accuracy	Found concentration (ng/ml)	% RSD	% Accuracy	Found concentration (ng/ml)	% RSD	% Accuracy
AML	100	98.7	1.3	98.70	99.3	1.7	99.30	101.2	1.8	101.2
	200	195.5	1.6	97.75	201.3	2.8	100.6	198.5	1.5	99.25
	300	295.6	2.1	98.53	296.3	1.9	98.76	298.5	1.3	99.50
FRS	800	798.1	2.6	99.76	795.2	3.2	99.40	791.5	2.3	98.93
	1600	1603.2	1.6	100.2	1597.5	1.4	99.84	1591.3	1.6	99.45
	2400	2398.5	1.8	99.93	2391.5	1.7	99.64	2392.5	1.2	99.69

Extraction Recoveries

Percentage extraction recoveries for AML and FRS were carried out using five replicates at three concentration level equivalent to HQC, MQC and LQC for both drugs and calculated by comparing the responses (peak areas) of both drugs from extracted samples to the responses of non-extracted standards Table-3. The % RSD of all three concentrations was within limits ≤ 15% and dose not changes with change in concentration.

Table 3. Extraction recovery data of AML and FRS (n=5)

Drug	Nominal concentration (ng/ml)	% Mean recovery ± SD	% RSD
AML	100	73.51±2.5	3.4
	200	75.19±2.1	2.7
	300	80.01±1.6	1.9
FRS	800	71.9±1.7	2.3
	1600	79.5±2.3	2.8
	2400	86.4±2.4	2.7

Stability of spiked plasma samples

Plasma samples were assessed at low and high concentration level i.e. 100 ng/mL, 300 ng/ mL and 800 ng/mL, 2400 ng/mL of AML and FRS respectively, for bench top and freeze thaw stabilities. Percentage change was calculated by comparing the peak area ratios of these samples with freshly prepared plasma samples of same concentration. The stability study

indicated acceptable variation in drug concentration over a span of 24 hours at room temperature. For bench top stability, changes were found 1.80-3.56 % and (-) 0.11-0.66% while for freeze thaw stability changes were found 0.73-2.30 % and 0.18-0.80 % for AML and FRS respectively Table-4. The % RSD for both drug were within limit.

Table 4. Stability of AML and FRS (n = 5)

Nominal concentration (ng/ml)	Mean found concentration [ng/ml (% change)]			SD	%RSD
	0 Hour	12 Hour	24 Hour		
Bench top stability of AML					
100	97.5 (2.50 %)	98.2 (1.80 %)	96.7 (3.30 %)	0.75	0.76
300	289.3 (3.56 %)	291.1 (2.96 %)	293.5 (2.16 %)	2.10	0.72
Bench top stability of FRS					
800	794.7 (0.66 %)	797.8 (0.27 %)	796.6 (0.42 %)	1.56	0.19
2400	2402.7 (-0.11 %)	2395.8 (0.17 %)	2397.4 (0.10 %)	3.61	0.15
Freeze thaw stability of AML	Day 0	Day 1	Day 2		
100	98.5 (1.50 %)	97.7 (2.30 %)	99.2 (0.80 %)	0.75	0.76
300	297.8 (0.73 %)	294.6 (1.80 %)	295.7 (1.43 %)	1.62	0.54
Freeze thaw stability of FRS					
800	798.5 (0.18 %)	794.9 (0.63 %)	793.6 (0.80 %)	2.53	0.31
2400	2389.3 (0.44%)	2386.9 (0.58 %)	2387.7 (0.51 %)	1.70	0.04

CONCLUSION

In conclusion, reported HPLC method involves simple single step rapid extraction procedure, economic isocratic mobile phase, and single detection wavelength for simultaneous estimation of analytes (226 nm). The run time was less than 11 minutes which allows minimal mobile phase consumption with analysis of a large number of plasma samples in a short time period. The method has been validated as per USFDA guidelines for bioanalytical methods and found to be linear, accurate and precise both in upper and lower concentration range i.e. 100 ng/mL, 300 ng/mL and 800 ng/mL, 2400 ng/mL of AML and FRS respectively with acceptable error and % RSD values were far less than 15.

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