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DEVELOPMENT AND VALIDATION OF RP-HPLC METHODS FOR SIMULTANEOUS ESTIMATION OF AMILORIDE & FUROSEMIDE IN BULK DRUG AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A new reverse phase high performance liquid chromatography method for the simultaneous estimation of amiloride and furosemide in tablet formulation is developed. The determination was carried out on a lichrospreRP-60selectB (4.6mm X250mm) column using a mobile phase of 0.05M potassium dihydrogen ortho-phosphate: acetonitrile (60:40 v/v, pH 3.5). The flow rate was 1.0 ml/min with detection at 283 nm. The retention time for amiloride was 2.53 min and for furosemide 8.01 min. amiloride and furosemide showed a linear response in the concentration range of 5-30 µg/ml and 40-240µg/ml, respectively. The results of analysis have been validated statistically and by recovery studies. The mean recoveries found for amiloride was 100.78% and for furosemide was 99.82%. Developed method was found to be simple, accurate, precise and selective for simultaneous estimation of amiloride and furosemide in tablets.

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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 MeOHand slightly soluble in water, imparting acidic character to its solutions. The drug, availableas the dihydrate, behaves as a mild diuretic and acts blocking the Na+ channels in the latedistal tubules and collecting ducts. By increasing the loss of sodium and chloride ions whilereducing the excretion of potassium [1]. Furosemide (4-chloro-*N*-furfuryl-5-sulphamoylanthranilic acid, FRS) is a whiteor slightly yellow powder, practically insolublein water but sparingly soluble in methyl alcohol (MeOH) and soluble in aqueous alkalinesolutions. This drug is a potent diuretic that inhibits the reabsorption of electrolytes in theascending limb of the loop of Henle and also in the renal tubules. While FRS has no clinicallysignificant effect on carbonic anhydrase, it enhances water excretion, increasing loss ofsodium, chloride and potassium ions. The association of FRS and AML (Fig. 1) furnishes avaluable natriuretic agent with a diminished

kaliuretic effect, minimizing the risk of alkalosisin the treatment of refractory oedema associated with hepatic cirrhosis or congestive heart failure [1]. Both individual drugs are official in the USP 24 and the BP 98. Since AMLand FRS up various diuretics which are administered worldwide to humans, it isnecessary to develop a simultaneous determination of these compounds in different matrixes.FRS has been individually determined in pharmaceutical formulations by extractivespectrophotometry[2], also, in biological fluids and urine it has been determined by HPLC[3-6] and HPLC-mass spectrometric analysis [7]. AML on the other hand, has beenindividually 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 thedetermination of AML in presence of other drugs in pharmaceutical formulations [12-16]and in biological fluids [17]. In the case of determination of FRS together with otherdrugs, their determination has been reported in tablets and urine by HPLC-EC [18], bymicellar electrokinetic chromatography [19] and by HPLC [20, 21]. The simultaneousdetermination of AML and FRS together with other drugs has been reported inurine by screening of diuretics using isocratic reversed phase LC with micellar organicmobile 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 AML andFRS. A HPTLC [24] and UV [25, 26] methods has been described for determination ofboth drugs in pharmaceuticals and HPLC [27] method for biological fluids. Although atpresent it is easy to find commercial pharmaceutical formulations containing both drugs, theanalytical simultaneous determination has not been reported yet in the actual pharmacopoeia[28].

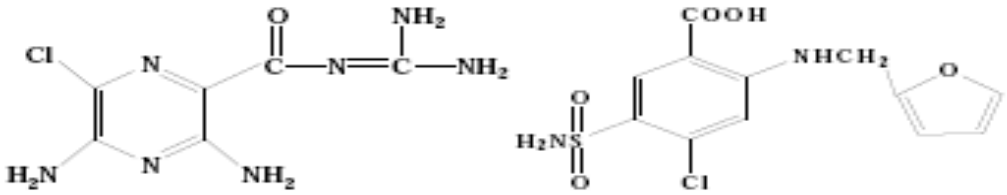

Fig. 1 Chemical structures of AML and FRS

OBJECTIVE

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 tablet formulation.

MATERIALS AND METHODS

Instrumentation

Liquid chromatographic system from dionex comprising of auto sampler injector, doublereciprocating plunger pump LC10 ATvp for constant flow and constant pressure delivery andu.v. detector connected to software chromeleon for controllingthe instrumentation as well as processing the data generated was used.

Reagents and chemicals

Amiloride (purity 100.0%) and furosemide (purity 100.0%) were obtained from wockhardtltd.L1chikalthana, Aurangabad.Maharastra (India). acetonitrile HPLCgrade were procured by finar Ltd., Mumbai. Potassium di-hydrogen ortho-phosphate was procured from Molychem Ltd.Indiathe 0.45-µm Nylon pump filter was obtained fromAdvanced Micro Devices (AmbalaCantt., India). Mili-Q water was used throughout the experiment. Otherchemicals used were of analytical or HPLC grade.

Chromatographic condition

The chromatography was performed by employing Lichrospere RP-60 select B (4.6 mm i.d × 250 mm) with UV detection at 283 nm. The isocratic mobile phase consisted of acetonitrile: 0.05M potassium di-hydrogen orthro-phosphate (40:60, v/v) of pH 3.5 adjusted with o-phosphoric acid. The flow rate was set at1.0 mL/min. Before use it was filtered through a 0.45- µm Nylon filterand degassed in an ultrasonic bath. The injection volume was10 µL. Peak homogeneity wasexpressed as peak purity and was obtained directly from the spectral analysis report obtainedby use of the chromeleon software.

Method development & validation

The developed method has been intensively validated as per Ich guidelines [29- 30],Using validation parameters viz System suitability, linearity, LOQ, accuracy, precision.LOQ is the minimumanalyteconcentration 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 wereprepared separately by dissolving appropriate amounts drugs in mobile phase. A homogenousmixed plasma stock of 5µg/mL and 40µg/mL of AML and FRS were prepared by spiking1.0 ml of respective standard stock solutions. Standard calibration solutions were prepared byfurther dilution of appropriate aliquots of standard stock solution with mobile phase to get final concentrations ranging from 5-30 µg/ml and 40-240 µg/ml of AML and FRSrespectively.

Sample preparation

Twenty tablets were weighed and average weight was calculated. The tablets were crushed to obtain fine powder. Tablet powder equivalent to 10.0 mg Amiloride and 80 mg of furosemide was transferred to 100.0 mL volumetric flask, added 50.0 mL mobile phase, sonicated for 20.0 min., volume was made up to the mark with mobile phase. The resulting solution was mixed and filtered through 0.45 nylon filter and filtrate was appropriately diluted to get final concentration of 5.0 µg/mL of Amiloride and 40.0 µg/mL of furosemide. The diluted solutions were filtered through 0.45 µm nylon filter to get clear solutions. The filtrate (10.0 µL) was injected into the column and chromatographed using optimized chromatographic conditions.

Specificity

The specificity of the RP-HPLC method evaluated by injecting blank solutions containing the mobile phase showed steady zero baseline at the selected wavelengths. Also the placebo chromatogram showed no additional peaks indicating the specificity of this method

Sensitivity

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

Precision

Repeatability:

To check the degree of repeatability of the method, six samples of the tablet formulations were analyzed as per the procedure given under Tablets. The standard deviation and % Relative Standard Deviation (% R.S.D.) were calculated.

Intermediate Precision (Intra-day and Inter-day precision)

The Intra and Inter-day precision was determined by assay of the sample solutions on the same day at different time intervals and on different days respectively. The S.D. and % R.S.D. were calculated

Accuracy (Recovery Studies)

The accuracy of the methods was assessed by recovery studies performed by the standard addition method. This technique involves the addition of standard drug solutions to pre-analysed sample solutions at 50%, 100%, and 150% level respectively. The mixed sample solutions were analyzed and the concentrations of both the drugs were determined under the optimized chromatographic conditions. Three determinations were performed at each level of recovery.

RESULTS AND DISCUSSION

Method development

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

Wavelength selection

AML exhibited two peaks at 214 and 362 nm, while FRS shows two absorptionmaxima at 230 and 274 nm (Fig. 1). Based on the spectral characteristics and overlayspectra 283 nm was selected as the wavelength for detection. Although it is not the absorptionmaxima of any of the drug but at this wavelength both the drugs show considerable absorption.

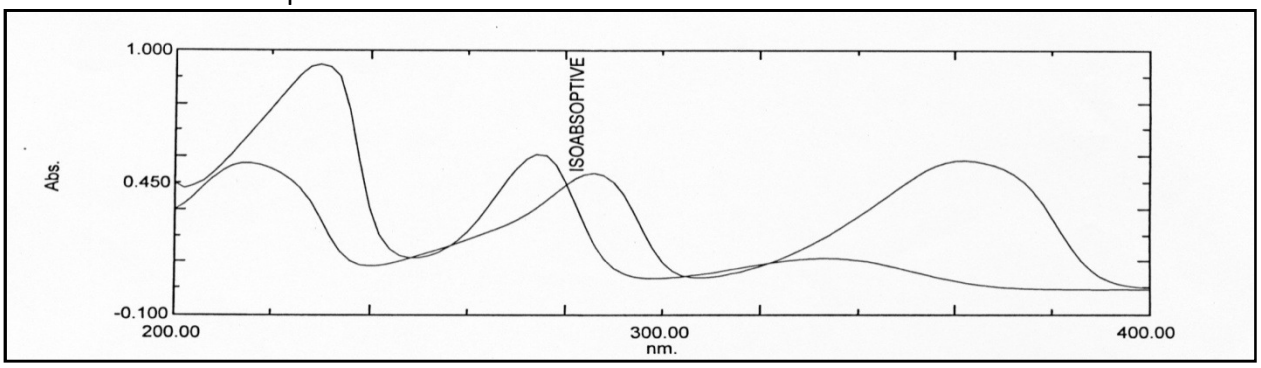

Fig.1 Representative Spectra of AML&FRS

Mobile phase selection

Taking into consideration of system suitability parameter like RT, Tailing factor, Number oftheoretical plates, HETP and other peak response like capacity factor, peak asymmetry andresolution of drug.The mobile phase containing varyingpercentagesof 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 mobilephase, in which peak of AML shows tailing effects. To consider pKa value of AML (8.7) andFRS (9.9), the neutral mobile phase at pH 7 consisting of methanol and water in the ratio of90:10 was used, FRS elutes at 14 min as broad peak Therefore, to reduce retention timealong with optimum resolution acidic mobile phase varying from pH 5.0 to 2.5 were tried. When methanol: phosphate buffer (60:40%v/v) was triedproblem with AML peak shape is observed. Then phosphate buffer is replaced with potassium di-hydrogen ortho-phosphate: acetonitrile with pH 3.5 which results in better resolution and sharp peaks so potassium di-hydrogen ortho-phosphate: acetonitrile, pH 3.5 (60:40%v/v) was selected as mobile phase(fig-2)

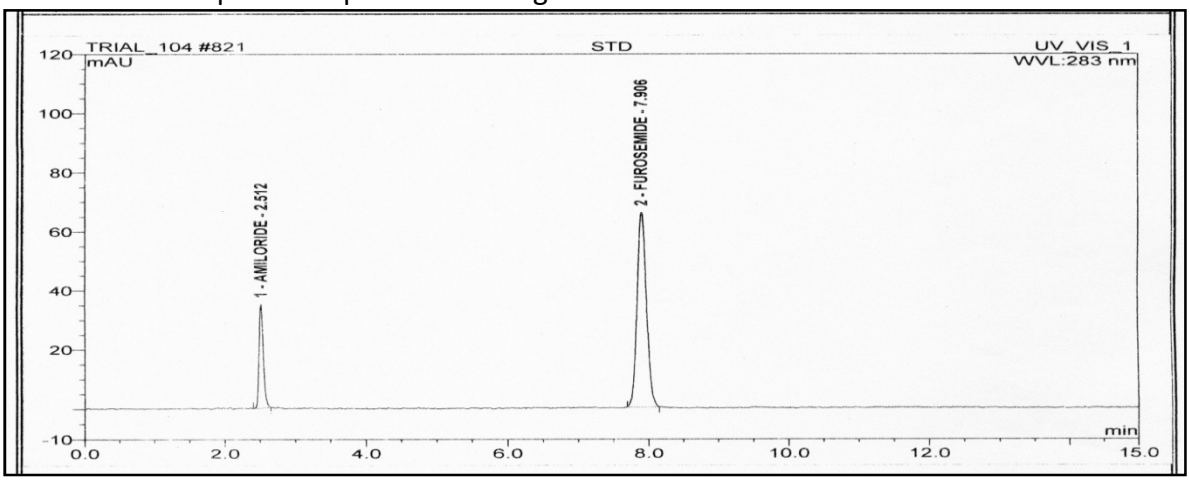

Fig.2 Representative Chromatogram ofAML and FRS

Flow rate selection

Flow rates between1.0 and 1.5 mL/ min was tried. At a flow rate of 0.5 ml peak offurosemide was broadened with longer retention of 14 min, 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 anoptimal signal-to-noise ratio with a reasonable separation time. Using a reversed-phase ODS column, the retention time was observed to be 2.53 and 8.01 for AML and FRS respectively,with a total run time of 15 min (Fig. 3).

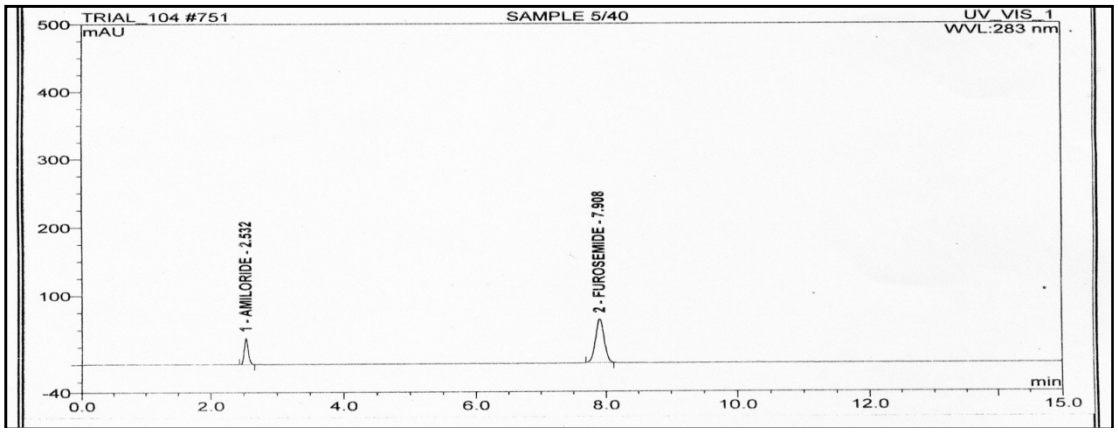

Fig. 3 Representative Chromatogram of AML and FRS

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 bothdrugs were injected separately and column performances like tailing factor, retention time (RT), resolution(R) andnumber of theoretical plates were observed (Table-1) and % RSD values for these parameters were found far less than 2%, which indicates acceptance of system performance

Table 1. System suitability parameters (n=6)

Sr. No.	Parameter	AML	S.D	%RSD	FRS	±S.D.	%RSD
1.	Retention Time (min.)	2.53	0.005	0.228	8.01	0.025	0.313
2.	Resolution (R)	2.8461					
3.	Tailing factor (T)	1.22	0.01	0.819	0.99	0.02	2.02
5.	No. of theoretical plates (N)	9855	19.67	0.1996	17879	23.71	0.13

Linearity and lower limit of quantitation (LLOQ)

Linearity was assessed using six different concentrations in three replicates. The method wasfound to be linear in concentration range of 5-30µg/ml and40:240 µg/ml for AMLand FRS respectively. The linear regression equations were found to be Y (AML) = 2.315 xconc. + 0.162 with (r2=0.999) and Y (FRS) = 9.144 x conc. + 1.419 (r2=0.999).The LLOQ were found0.00375 µg/ml and0.010 µg/ml forAML and FRS respectively. The LOD of the method was found to be 0.00124 µg/ml and 0.00528 µg/ml for AML and FRS Respectively.

Accuracy (Recovery)

Accuracy was determined by analyzing three dilutions of known concentration in threereplicates. The results of accuracy were expressed in terms of % nominal concentration and itwas observed for AML in between 100.58-101.39 % and for FRS in between 99.22- 100.42 %

Table-2.Accuracy (n=3)

Level of % Recovery	Amount taken (µg/mL)		Amount of standard added (µg/mL)		Total amount recovered (µg/mL)		% Recovery*	
	AML	FRS	AML	FRS	AML	FRS	AML	FRS
50	5	40	2.5	20	7.55	60.13	101.20	100.65
50	5	40	2.5	20	7.55	60.02	101.74	100.06
50	5	40	2.5	20	7.53	60.01	98.80	100.58
100	5	40	5	40	10.08	80.13	101.79	100.32
100	5	40	5	40	10.03	79.35	100.79	98.37
100	5	40	5	40	10.01	79.59	101.30	98.97
150	5	40	7.5	60	12.59	99.88	101.02	99.80
150	5	40	7.5	60	12.58	99.83	101.14	99.71
150	5	40	7.5	60	12.55	100.02	101.83	100.03

Level of % Recovery	% Mean Recovery*		± S.D.		% R.S.D.	
	AML	FRS	AML	FRS	AML	FRS
50	100.58	100.42	1.564	0.317	1.554	0.3156
100	101.29	99.22	0.500	0.998	0.493	1.0058
150	101.39	99.85	0.382	0.1652	0.376	0.1654

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 three replicates wereanalyzed in same day for repeatability and % RSD for the both drugs were found far less than2% (Table-3,4), which is acceptable limit of the developed analytical method.

Intermediate Precision

Day-to-day and analyst-to-analyst variation was analyzed using three dilutions in threereplicates on three different days with three analysts. Although % RSD value for AML is higherthan FRS, but all the results of both drugs fall within acceptable limits (Table-5, 6)

Table 3. Intraday precision of AML (Repeatability)

Conc(µg/ml)	%ASSAY			MEAN %ASSAY	± S.D.	%R.S.D
	TRIAL 1	TRIAL2	TRIAL3			
5	98.63	99.48	99.37	99.16	0.462	0.465
10	99.01	99.28	99.52	99.27	0.251	0.252
15	99.05	99.21	99.55	99.27	0.255	0.256

Table4. Intraday precision of FRS (Repeatability)

Conc(µg/ml)	%ASSAY			MEAN %ASSAY	± S.D.	%R.S.D
	TRIAL 1	TRIAL2	TRIAL3			
40	99.36	99.65	99.55	99.52	0.147	0.148
80	99.56	99.51	99.59	99.55	0.040	0.040
120	99.58	99.61	99.50	99.56	0.056	0.057

Table 5.Interday precision of AML (Intermediate)

Conc (µg/ml)	%ASSAY			MEAN %ASSAY	± S.D.	%R.S.D
	DAY 1	DAY2	DAY3			
5	98.15	99.74	99.38	99.08	0.832	0.837
10	99.26	99.35	99.44	99.35	0.090	0.090
15	99.37	99.44	99.68	99.49	0.162	0.163

Table 6.Interday precision ofFRS (Intermediate)

Conc(µg/ml)	%ASSAY			MEAN %ASSAY	± S.D.	%R.S.D
	DAY 1	DAY2	DAY3			
40	98.99	99.48	99.50	99.32	0.346	0.348
80	99.48	99.80	99.59	99.59	0.181	0.181
120	99.53	99.53	99.54	99.53	0.005	0.005

Robustness

Robustness for RP-HPLC method was determined by analysis of samples under deliberately changed chromatographic conditions. The flow rate of the mobile phase was changed from 1.1 ml/min to 1 ml/min and 1.2 ml/min. The temperature was changed by ± 2%. The effect on retention time and peak parameter were studied. Result for robustness studies are given in(Table-7)

Table7. Result For Robustness Studies

Inj	Flow Rate ml/min	Ret Time AML(min)	Ret Time FRS(min)	%Content AML	%Content FRS
1	1	2.53	7.89	99.35	99.81
2	0.9	2.58	8.01	99.93	99.31
3	1.1	2.47	7.62	100.41	99.31
Mean		2.52	7.84	99.89	99.47
± S.D		0.055	0.199	0.530	0.288
%R.S.D.		0.712	2.538	0.288	0.289
Inj	**Temp^{0}C**	**Ret Time AML(min)**	**Ret Time FRS(min)**	**%Content AML**	**%Content FRS**
1	40	2.53	7.90	99.55	99.62
2	39.9	2.52	7.89	99.84	98.77
3	40.1	2.54	7.89	100.1	99.30
Mean		2.53	7.89	99.83	99.08
± S.D.		0.01	0.005	0.275	0.429
%R.S.D.		0.395	0.072	0.275	0.432

DISCUSSION:

It is evident from the study of RP- HPLCmethod that the newly developed method can be used for routine analysis as an alternate method for the simultaneous estimation of AML and FRS in bulk and tablet dosage form. But with certain limitations that only HPLC grade solvents are to be used for the experimental works. All the preparations have to be degassed and micro filtered before injection into the column. Purging, flushing and priming are necessary, both before and after the completion of experimental works.

CONCLUSION

In conclusion, reported HPLC method involves simple, precise, accurate, economic with isocratic mobile phase, and single detection wavelength for simultaneousestimation of analytes (283 nm). The run time was less than 15 minutes which allowsminimal mobile phase consumption with analysis of a large number of samples in ashort time period. The method has been validated as per Ich guidelines for analyticalmethods and found to be linear, accurate and precise both in upper and lower concentration range i.e. 5 µg/ml,

10µg/mland 40 µg/ml, 80 µg/mlof AML and FRS respectively with acceptable error and % RSD values were far less than 2%.

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