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Development and Validation of RP-HPLC Method for the Simultaneous Determination of Amiloride Hydrochloride and Furosemide in Pure and Pharmaceutical Dosage Form

Jinal N. Tandel
Sardar Patel College of Pharmacy, INDIA

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ABSTRACT
A simple, selective, rapid, precise and economical reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Amiloride Hydrochloride and Furosemide from pharmaceutical formulation. The method was carried out on an Enable C_{18} (250x4.6) mm; 5 µm with a mobile phase consisting of acetonitrile: Water (adjusted to pH 4 using orthophosphoric acid) in the ratio of 70:30 v/v at a flow rate of 1.0 ml/min. Detection was carried out at 281nm. The retention times of Amiloride Hydrochloride and Furosemide were 2.21 min. and 7.60 min., respectively. The developed method was validated according to ICH guidelines for evaluation of accuracy, precision, linearity, limit of detection, limit of quantitation and robustness. The proposed method can be used for the estimation of these drugs in combined dosage form.
Keywords: RP-HPLC, amiloride hydrochloride (AML), furosemide (FUR)

INTRODUCTION

Furosemide (FUR) is chemically 4-chloro-2-furfurylamino-5-sulphamoyl benzoic acid **(Figure 1)**. It is a potent loop diuretic [1]. It acts primarily by blocking sodium and chloride reabsorption in the ascending limb of the loop of Henle. FUR helps to conserve potassium and minimize the risk of alkalosis, in the treatment of oedema associated with hepatic cirrhosis and congestive heart failure.

Amiloride hydrochloride (AML) is chemically 3,5-diamino-N-(diaminomethylene)-6-chloropyrazinecarboxamide monohydrochloride dihydrate **(Figure 2).** It is a potassium sparing diuretic [1].

AML in conjunction with loop diuretics such as FUR, reduces overall fluid volume in the body and help to control symptoms of heart disease, kidney and liver disease [2]. In recent years, these two drugs are successfully used in association in the treatment of many diseases related to kidney, liver and heart and the pharmaceutical preparation containing both drugs

Correspondence: Jinal N. Tandel, *Department of Quality Assurance, Sardar Patel College of Pharmacy, Bakrol, Anand, Gujarat, India.*
jinaltandel1202@gmail.com

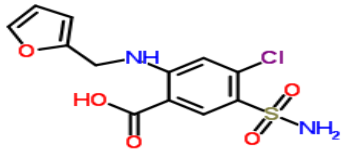

Figure 1. Chemical structure of Furosemide (FUR)

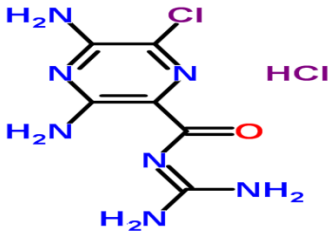

Figure 2. Chemical structure of Amiloride hydrochloride (AML)

have been marketed. Literature review showed that both AML and FUR in bulk and tablet dosage form is official in IP, 2010 [3], and USP, 2007 [4]. Several analytical methods have been reported for estimation of AML and FUR with other drugs which include spectrophotometry [5, 6], HPLC [7-11], HPTLC [12]. Only RP-HPLC method has been reported for simultaneous estimation of AML with FUR in tablet dosage forms [13]. In the present work, a successful attempt has been made to estimate both these drugs simultaneously using RP- HPLC method with different mobile phase composition than the published one. This study attempts to develop a simple, accurate and precise analytical chromatographic method, which can quantify these drugs simultaneously from a combined tablet dosage form. The developed method was validated as per ICH guidelines and found to comply with the acceptance Criteria [14].

EXPERIMENTAL SECTION

Materials and Reagents

Acetonitrile (HPLC grade) and Distilled water (HPLC grade) ware procured from Fisher scientific pvt. Ltd (India), orthophosphoric acid (AR grade) also procured from Fisher scientific pvt. Ltd (India). Reference standards of AML and FUR were obtained as a gift sample from Vapi care pharma, Vapi. (India). Marketed dosage form AMIFRU-40 (AML 5mg & FUR 40mg) was procured from local market, VMS Medical Store Vapi, India.

Apparatus and chromatographic conditions

Chromatographic separation was performed on a model of Simadzu LC-2010 HT containing uv detector and LC solution software. A Enable C_{18} (250x4.6) mm; 5 µm was used for the separation, mobile phase of a mixture of acetonitrile and water (adjusted to pH using

orthophosphoric acid) in the ratio of 70:30 v/v was delivered at a flow rate of 1.0 ml/min with detection at 281 nm. The mobile phase was filtered through a 0.45 µm membrane filter and degassed. The injection volume was 10 µl; Analysis was performed at ambient temperature.

Preparation of standard solutions

Standard stock solution of AML (100 µg/ml) and Standard stock solution of FUR (1000 µg/ml) was prepared by using mobile phase. From the standard stock solution, mixed standard solution was prepared to contain 25 µg/ml of AML and, 200 µg/ml of FUR was prepared with mobile phase.

Preparation of sample solution

Tablets powder equivalent to 200 mg FUR and 25 mg AML was weighed and dissolved in 100 ml mobile phase. The solution was sonicated for 15 min and was filtered through a Whatman filter paper no. 45. Further dilutions were made to get a concentration of 200 µg/ml of FUR and 25 µg/ml of AML. These solutions were filtered through 0.45 µ membrane filter.

Calibration Curve of Amiloride Hydrochloride and Furosemide

Calibration curves were prepared by taking appropriate aliquots 1, 2.5, 4, 5.5, 7, 8.5 ml of working standard solution of AML and 0.8, 2.0, 3.2, 4.4, 5.6, 6.8 ml of standard solution FUR in 10 ml vol. flask and dilute up to the mark with mobile phase of to give 10-85 µg/ ml of AML and 80-680 µg/ ml of FUR. The standard solution was run for 12 minutes using mobile phase at a flow rate of 1ml/min. The graph of peak area vs concentration was plotted, regression equation and correlation co- efficient for both drugs were obtained.

Chromatographic separation

Standard solutions of 10-85 µg/ml of AML and 80-680 µg/ml FUR were injected in column with injection volume 10 µl. The chromatogram was run for appropriate minutes with mobile phase Acetonitrile: water (70:30v/v). The detection was carried out at wavelength 281 nm. The chromatogram was stopped after separation achieved completely. Data related to peak like area, height, retention time, resolution etc. maintained.

System Suitability Test

It is an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use.

Formulas for calculation of SST are:

1) Resolution:

$$\boldsymbol{R_s = \frac{tR_2 - tR_1}{0.5(w_1 + w_2)}}$$

where R_s is resolution, tR_1 and tR_2 are the retention times of components 1 and 2, w_1 and w_2 are peak width of components 1 and 2.

2) Theoretical plate:

$$\boldsymbol{N = 16\left(\frac{tR}{w}\right)^2}$$

where N is the number of the theoretical plate, tR is the retention time, w is the peak width.

3) Tailing factor

$$\boldsymbol{T = W0.05/2f}$$

where T is tailing factor, $W0.05$ is the width of the peak at 5 percent height, and f is distance at 5 percent height.

Method Validation

Specificity

Specificity is a procedure to detect quantitatively the analyte in the presence of components that may be expected to be present in the sample matrix. Specificity of developed method was established by spiking of AML and FUR in hypothetical placebo (i.e. might be expected to be present) and expressing that analytes peak were not interfered from excipients.

Linearity

Aliquots of standard solutions of AML and FUR in range 10-85 g/ml and 80-680 g/ml respectively, were prepared from working standard solution and injected to system with stated chromatographic conditions and analyzed. The graph of peak area obtained versus respective concentration was plotted. The mean area with its standard deviation and % relative standard deviation of peak were calculated.

Precision

Precision of the methods was determined by performing interday variation, intraday variation and method repeatability studies. In interday variation, the peak area of standard solutions of AML (10, 25, and 40 µg/ml) and FUR (80, 200, and 320 µg/ml) were measured on three consecutive days. In intraday variation the peak area was measured three times in a day. In repeatability study, six concentrations of both the drugs AML (25 µg/ml) FUR (200 µg/ml) were analysed.

Recovery studies

To study the accuracy of the proposed method, recovery studies were carried out by standard addition method at three different levels. Known amount of the two drugs was added to pre-analyzed tablet powder and percentage recoveries were calculated.

Limits of detection and Quantification

According to ICH, limit of detection (LOD) is the lowest concentration of the analyte that can be detected and limit of quantification (LOQ) is the lowest concentration of analyte that can be detected with acceptable accuracy and precision. LOD and LOQ are calculated from the formulae $3.3\sigma/s$ and $10\sigma/s$ respectively. Where σ is the standard deviation of y-intercepts of the regression line and s is the slope of the calibration curve.

Robustness

It should show the reliability of an analysis with respect to deliberate variations in method parameters.

In case of liquid chromatography, examples of typical variations are

- Influence of variations of pH(±0.2) in a mobile phase
- Flow rate. (±0.2)
- Wavelength (±2%).

System suitability

System suitability was established in order to determine the adequate resolution and reproducibility of the proposed method. Suitability parameters including retention factor, resolution, asymmetry factor, and plate number were investigated.

Assay of the marketed formulation

The developed method was applied to the simultaneous determination of AML and FUR in pharmaceutical formulations. Sample was analyzed by performing six independent determinations and each series was injected in triplicate.

RESULTS AND DISCUSSION

Selection of elution mode

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. Hence C18, 250 × 4.6 mm column of 5 µm particle packing was selected for separation of AML and FUR. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

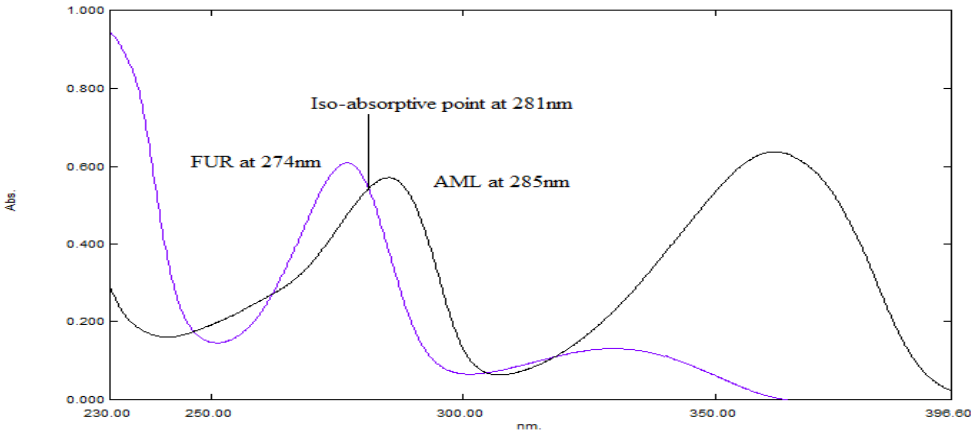

Figure 3. Overlay UV Spectrum of Amiloride Hydrochloride and Furosemide showing selection of wavelength detection

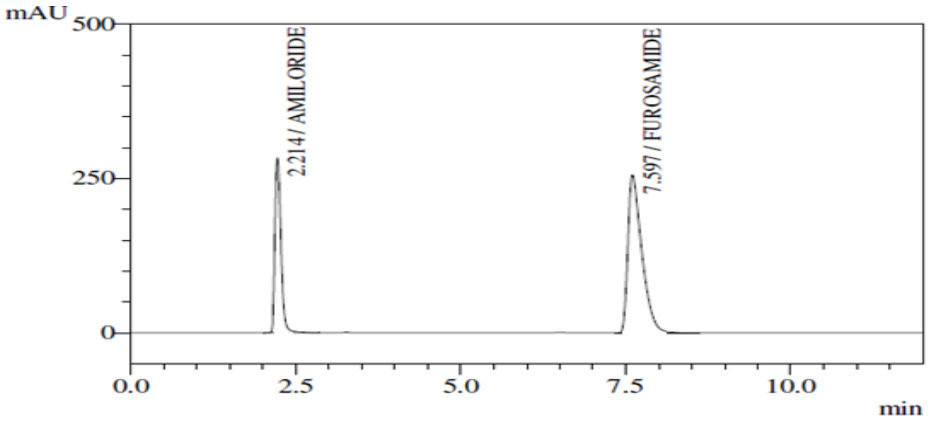

Figure 4. Typical chromatogram for the standard solution of AML and FUR

Selection of wavelength

Both AML and FUR show reasonably good response at 281nm. Mention in **Figure 3**.

Mobile phase optimization

Chromatographic parameters were optimized to develop a HPLC method for simultaneous determination of AML and FUR with short analysis time (< 10 min), and acceptable resolution (RS>2). Various compositions of mobile phases like methanol: water and acetonitrile (ACN) : water in different ratios were tried. But with mixed ACN: Water in the ratio of (70:30 v/v, pH adjusted to 4 with 1% Orthophosphoric acid) at a flow rate of 1 ml/min, symmetrical peaks with good resolution were obtained. The optimum wavelength for detection was set at 281 nm at which better detector response for both drugs was obtained. The retention times were 2.21 and 7.59 min for AML and FUR respectively **(Figure 4)**.

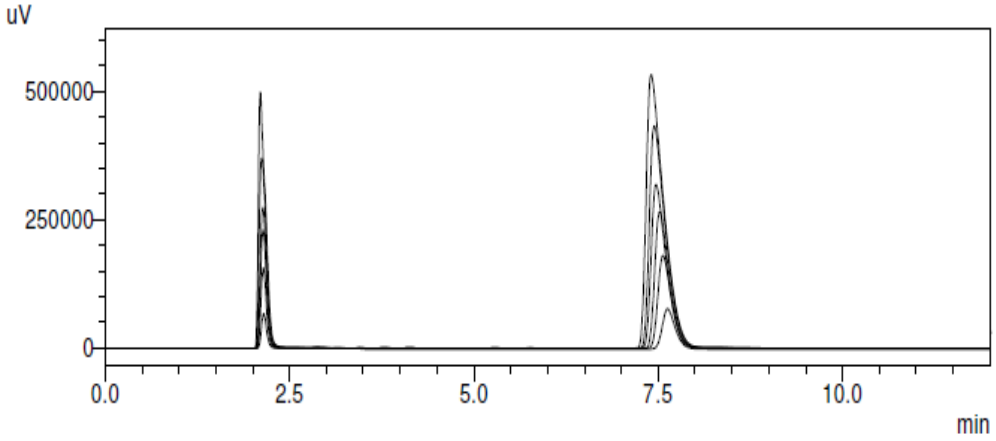

Figure 5. Overlay chromatogram of different concentrations of binary mixtures of AML and FUR

Table 1. Statistical data of AML and FUR

Sr.No.	Parameter	AML	FUR
1.	Linearity range (µg/ml)	10-85	80-685
2	Correlation co-efficients	0.9993	0.9994
3	Regression line equations	36644x-77053	9997.9x-106739
4.	Limit of Detection (µg/ml)	1.88853	11.09421
5.	Limit of Quatificatiion (µg/ml)	5.722819	33.61883

Validation

Calibration graphs were constructed by plotting the peak area versus their corresponding concentrations **(Figure 5).** Good linearity was obtained in the range of 10-85 µg/ml and 80-680 µg/ml for AML and FUR, respectively. The results are shown in **Table 1**. LOD and LOQ were calculated from the slope and standard deviation of y-intercepts of the regression line of the calibration curve. The results are shown in **Table 1**. The precision of the method and instrument precision was evaluated and relative standard deviation (RSD) values were calculated. The RSD values for AML and FUR showed that the precision of the method was satisfactory. The results are shown in **Table 2**. The accuracy of the method was determined by recovery studies. The recoveries were close to 100% for AML and FUR. The results are shown in **Table 3**. Developed method was found to be robust when the mobile phase ratio, flow rate and pH was changed. The results are shown in **Table 4**. SST parameters were shown in **Table 5**.

Table 2. Precision parameters for AML and FUR

Parameter		AML	FUR
Repeatability n=6	**%RSD**	1.73	0.94
Interday precision (n=3)	**%RSD**	0.20-0.54	0.14 -1.58
Intraday precision (n=3)	**%RSD**	0.16-0.47	0.12-1.03

Table 3. Accuracy data of AML and FUR

Drug	Level	Amount of sample taken (µg/ml)	Amount of standard spiked (µg/ml)	Total Conc. Found (µg/ml)	% Recovery ± S.D. (n=3)
AML at 281 nm	80 %	25	20	45.48	101.07 ±0.0802
	100 %	25	25	50.94	101.28 ± 1.1269
	120 %	25	30	56.09	101.99 ± 0.4309
FUR at 281 nm	80 %	200	160	360.74	100.20 ± 0.2715
	100 %	200	200	407.88	101.97 ± 0.6678
	120 %	200	240	440.33	100.07 ± 0.2351

Table 4. Robustness study data

	Parameter	AML	FUR
	pH(+0.2units)	0.62	0.22
	pH(-0.2units)	0.37	0.13
Robustness %RSD	Flow rate (+0.2 units)	0.42	0.18
	Flow rate (-0.2 units)	0.35	0.09
	Wavelength (+2%)	0.29	0.1
	Wavelength (2%)	0.55	0.06

Table 5. System suitability parameter

FACTOR	AML	FUR
Retention time (min)	2.21	7.59
Tailing factor	1.49	1.85
Theoretical plates	2830	6545
Resolution	9.81	

Assay of marketed formulation

The tablet powder equivalent to 40 mg FUR was taken in 100 ml volumetric flask so it contains 5 mg AML in it. That contents is diluted with mobile phase and mark up to 100 ml with same solution. It gives 400 (µg/ml) of FUR and 50 (µg/ml) of AML. The prepared solution was filtered through 0.45 micro membrane filter. The diluted solution was analyzed under optimized chromatographic conditions. The areas of resulting peak were measured at 281 nm. Shown in **Table 6**.

Table 6. Analysis of Marketed Formulation

Batch no. of Tablet	Actual concentration µg/ml		Amount obtained µg/ml		% Assay of AML ± S.D. (n=3)	% Assay of FUR ± S.D. (n=3)
	AML	FUR	AML	FUR		
NA0091029	5	40	4.97	40.2	99.98 ± 0.2750	100.52 ± 0.1734
NA0092099	5	40	4.99	39.98	99.80 ± 0.2100	99.98 ± 0.1350
NA0093023	5	40	4.98	40.15	99.67 ± 0.1474	100.37 ± 0.0493

Table 7. Comparison between reported and proposed method

Specification	Reported method	Proposed method
Column	HIQ SIL C18 (250×4.6 mm, 5 µm)	Enable C_{18}(250x 4.6)mm ; 5µm
Mobile phase	50 mM phosphate buffer pH 3.0 and acetonitrile (50:50)	Acetonitrile : water of (70:30 v/v) pH 4
Concentration range	FUR - 80-160 µg/ml AML - 10-20 µg/ml	AML- 10-80 µg/ml FUR – 80- 640 µg/ml
Retention time	FUR - 3.038 min AML - 10.002 min	AML – 2.20 min FUR – 7.61 min
Resolution	6.85 min.	9.81 min.
LOD	Data was not found	AML- 1.88 µg/ml FUR – 11.09 µg/ml
LOQ	Data was not found	AML- 5.72 µg/ml FUR – 33.61 µg/ml

Comparison between proposed method and reported method

According to literature review there was one HPLC method is reported for the estimation of the AML and FUR. The Comparison between proposed RP-HPLC method and reported RP-HPLC method was shown in **Table 7**.

CONCLUSION

The proposed sensitive RP-HPLC method gives accurate and precise results for determination of AML and FUR in marketed formulation (tablet) without prior separation and is easily applied for routine analysis. The most striking feature of the method is its simplicity and rapidity. Method validation has been demonstrated by variety of tests for linearity, accuracy, precision, LOD, LOQ and robustness. The proposed method was successfully applied to determination of these drugs in commercial tablets.

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