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STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF FRUSEMIDE AND AMILORIDE HYDROCHLORIDE IN TABLET DOSAGE FORM

Renu Solanki*

Lachoo memorial College of Science and Technology, Pharmacy Wing, Jodhpur, Rajasthan, India

ABSTRACT

This article focuses on stability indicating RP-HPLC method for simultaneous estimation of Frusemide and Amiloride Hydrochloride as API and in tablet dosage form, validation of developed method and its application in pharmaceutical companies. Acetonitrile, potassium di hydrogen phosphate and phosphoric acid were used. Chromatographic conditions comprised of C_{18} column (250 × 4.6 mm, 5 µm), mobile phase of phosphate buffer pH 3.0 and acetonitrile in 50:50 ratio, flow rate at 1 ml/min, ultraviolet detection at 283 nm. The retention time of Frusemide was found to be 3.038 min. and Amiloride Hydrochloride was 10.002 min. respectively. The linear regression analysis for calibration plots showed correlation coefficient, r = 0.99995 at concentration range of 20 to 200 µg/ml for Frusemide and *r* = 0.99925 at concentration range of 10 to 100 µg/ml for Amiloride hydrochloride. The stability studied indicated that the drugs are susceptible to wet heat, dry heat, day light, acidic and alkaline conditions with maximum degradation in oxidation degradation. Statistical analysis proved the developed RP-HPLC method as simple, reproducible and selective for the estimation of Frusemide and Amiloride hydrochloride in tablet dosage form and it can be employed as stability-indicating method.

***Keywords*:** Chromatograms; validation; degradation products; stability method

1. Introduction:

Frusemide (Fru) is chemically 4-chloro-2-furfurylamino-5-sulphamoyl benzoic acid. 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. Fru 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 (Ami) is 3,5-diamino-N-(diaminomethylene)-6 chloropyrazinecarboxamide monohydrochloride dehydrate. It is a potassium sparing diuretic[1]. Ami in conjunction with thiazide loop diuretics such as Fru, reduces overall fluid volume in the body and help to control symptoms of heart disease, kidney and liver disease[2,3]. Both drugs are official in IP[4,7], BP[5,8] and USP-NF[6,9] .

Several analytical methods have been reported for quantitative determination of frusemide individually by UV[10,11], GC[12], TLC[13], HPLC[14,15] and Colorimetry[16,17] and quantitative determination of amiloride hydrochloride is carried out by UV[18,19], TLC[20] and HPLC[21] methods. Although, many methods have been reported in the literature for the estimation of Fru and Ami individually, but there is no stability indicating high-performance liquid chromatography (HPLC) method for their simultaneous determination in pharmaceutical tablet dosage form.

Corresponding Author*: solankirenu@yahoo.com

The ICH guideline requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substances[22].An ideal stability-indicating method is one that quantifies the drug *per se* and also resolves its degradation products.

The aim of the present work was to develop an accurate, specific, reproducible and stability indicating method for the determination of frusemide and amiloride hydrochloride in the presence of its degradation products and related impurities as per ICH guideline[23].

2. Materials:

2.1.Reagents: Working reference standards of frusemide and amiloride hydrochloride were supplied by Elder Pharmaceuticals ltd., Mumbai.The marketed tablet formulation Amifru (batch no. 0605004, Elder Pharmaceuticals) was procured from the market. Acetonitrile (HPLC grade), potassium di hydrogen ortho phosphate (AR grade) and ortho phosphoric acid (AR grade) were used for mobile phase preparation.

2.2. Apparatus: A gradient HPLC (Water, Germany) with PU–1580 double reciprocating pump, UV–1575 UV detector, and RP-C18 column (5 µm particle size) was used. The RP-HPLC system was equipped with winchrom software for data processing. Method was developed using a HIQ SIL, C18 (250 X 4.6 mm, 5 µm) column with a flow rate of 1 ml/min

3. Methods:

3.1. Chromatographic conditions: Chromatographic separation was achieved at 26˚C on a reversed phase column using a mobile-phase consisting of acetonitrile and 50 mM phosphate buffer (pH of 3.0 ± 0.05) in the ratio of 50:50 v/v. The flow rate was kept at 1 ml/min and detection was performed at 283 nm. The injection volume was 10 µl. Standard solution containing frusemide and amiloride hydrochloride were prepared from stock solution by suitable dilution to get a concentration of 200 µg/ml and 100 µg/ml respectively.

3.2. Diluent preparation: Mobile phase consisting of acetonitrile and phosphate buffer (pH of 3.0 ± 0.05) in the ratio of 50:50 v/v was used as the diluent.

3.3. Stock solutions preparation of frusemide and amiloride hydrochloride: Frusemide (20 mg) was accurately weighed and transferred to 100 ml volumetric flask; 50 ml of diluent was added to it and the final volume was made upto 100 ml mark using the same solvent. The solution was filtered through 0.45 µm membrane filter. The solution was sonicated for 10 min for degassing. The final solution contained 200 µg/ml of Frusemide.

Amiloride hydrochloride (10 mg) was accurately weighed and transferred to 100 ml volumetric flask; 50 ml of mobile phase was added to it and final volume was made upto 100 ml mark using the same solvent. The solution was filtered through 0.45 µm membrane filter. The solution was sonicated for 10 min for degassing. The final solution contained 100 µg/ml of Amiloride Hydrochloride.

3.4. Standard preparation of frusemide and amiloride hydrochloride: Standard solution of frusemide (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 ml) was pipetted out in a series of ten, 10 ml volumetric flasks respectively. The volume in each flask was adjusted to 10 ml mark with mobile phase and mixed the contents so as to obtain a final concentration in the range of 20 to 200 µg/ml. The solution was filtered through 0.45 µm membrane filter. The solution was then sonicated for 10 min for degassing. This final solution contains 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml of Frusemide. The filtered solution was injected into the HPLC system. The chromatogram is shown in figure 1.

Standard solution of amiloride hydrochloride (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml) was pipetted out in a series of ten, 10 ml volumetric flasks respectively. The

volume in each flask was adjusted to 10 ml mark with mobile phase and mixed the contents so as to obtain a final concentration in the range of 10 to 100 µg/ml. The solution was filtered through 0.45 µm membrane filter. The solution was then sonicated for 10 min for degassing. This final solution contains 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml of Frusemide. The filtered solution was injected into the HPLC system. The chromatogram is shown in figure 2.

3.5. Synthetic mixture preparation of frusemide and amiloride hydrochloride: The API mixture of frusemide and amiloride hydrochloride was prepared in the ratio of 8:1. The decision of this ratio of drugs in the API mixture was based upon the dosage strength of combination, which is available in the market. Accurately weighed 80 mg of frusemide and 10 mg of amiloride hydrochloride were transferred to100 ml volumetric flask, dissolved and diluted to 100 ml with mobile phase. The solution was filtered through 0.45 µm membrane filter. The solution was then sonicated for 10 min for degassing. The filtered solution was injected into the HPLC system. The chromatogram is shown in figure 3.

3.6. Accelerated degradation study of synthetic mixture of frusemide and amiloride hydrochloride solution

3.6.1. Wet heat degradation: 100 ml of synthetic mixture of frusemide and amiloride hydrochloride solution was refluxed on a water bath for 1 hour at 60°C. Then the solution was cooled upto room temperature. The solution was filtered through 0.45 µm membrane filter and was sonicated for 10 min. for degassing. The solution was immediately injected into the HPLC system and was then analyzed.

3.6.2. Dry heat degradation: The drugs were stored in an oven at 105°C for 1 hour. From this accurately weighed frusemide and amiloride hydrochloride in the ratio of 8:1 was transferred to 100 ml volumetric flask, added 50 ml of mobile phase and sonicated to dissolve. Then the volume was adjusted to 100 ml with the mobile phase. The solution was filtered through 0.45 µm membrane filter and was sonicated for 10 min. for degassing. The solution was immediately injected into the HPLC system and was then analyzed.

3.6.3. Photochemical degradation: 10 ml of synthetic mixture of frusemide and amiloride hydrochloride solution was exposed to direct sunlight for 17 hours. The solution was filtered through 0.45 µm membrane filter and was sonicated for 10 min. for degassing. The solution was immediately injected into the HPLC system and was then analyzed.

3.6.4. Oxidative degradation: To 10 ml of synthetic mixture of frusemide and amiloride hydrochloride solution, 10 ml of 3 % v/v hydrogen peroxide was added and the solution was shaken. The mixture was allowed to stand for 6 hours. The solution was filtered through 0.45 µm membrane filter and was sonicated for 10 min. for degassing. The solution was immediately injected into the HPLC system and was then analyzed.

3.6.5. Acidic degradation: To 10 ml of stock solution of synthetic mixture of frusemide and amiloride hydrochloride solution, 10 ml of 0.1 N hydrochloric acid was added. This solution was refluxed on water bath for 8 hours at 60°C. Then the solution was cooled to room temperature. The resulting solution was neutralized by 0.1 N sodium hydroxide solution, to avoid any interference of acid. The solution was filtered through 0.45 µm membrane filter and was sonicated for 10 min. for degassing. The solution was immediately injected into the HPLC system and was then analyzed.

3.6.6. Alkaline degradation: To 10 ml of synthetic mixture of frusemide and amiloride hydrochloride solution, 10 ml of 0.1 N sodium hydroxide was added. This solution was refluxed on water bath for 8 hours at 60°C. Then the solution was cooled to room temperature. The resulting solution was neutralized by 0.1 N

hydrochloric acid solution, to avoid any interference of base. The solution was filtered through 0.45 µm membrane filter and was sonicated for 10 min. for degassing. The solution was immediately injected into the HPLC system and was then analyzed.

4. Results:

4.1. **Method development:** The chromatographic conditions were optimized with a view to develop a stability-indicating assay method. It included mobile phase of acetonitrile and phosphate buffer (pH of 3.0 ± 0.05) in the ratio of 50:50 v/v at the detection wavelength of 283 nm, acquisition time of 15 min, injection volume of 10 µl, flow rate of 1.0 ml / min and column HIQ SIL, 250 X 4.6, RP-C_{18} with 5µm at a temperature of 26º C. Except acetonitrile: phosphate buffer (50:50), all the other mobile phases like pure methanol, methanol: phosphate buffer (50:50), (90:10) showed too much signal to noise ratio (asynchronous and synchronous noise), baselines were not proper (there was a noisy baseline, drifted baseline and cyclic baseline). At pH = 3.0 ± 0.05, the peaks of both the drugs got separated and were identified easily which was not in the case at pH = 11.0 ± 0.05 (three different size peaks were observed in the retention time ranging from 2 to 4 min. and they were not identified easily) while at pH = 6.0 ± 0.05, out of three peaks between 2 to 4 min. one peak got eliminated, second peak got very much decreased in size and the third peak persist, showed slight drifting. Table 1 represents the system suitability parameters for the method.

4.2. **Calibration curves:** From the calibration curve data, the linear regression equation and correlation coefficient for frusemide was found to be $y = 8930.7x - 16728$, $R^2 = 0.9999$ and $r = 0.99995$. The method was found to be linear in the range of 20 to 200 µg/ml for frusemide while for amiloride hydrochloride it was found to be $y = 10555x + 12633$, $R^2 = 0.9985$ and $r = 0.99925$, where y is peak area, x is the concentration of drug solution and r is the correlation coefficient respectively. The method was linear in the range of 10 to100 µg/ml for amiloride Hydrochloride.

4.3. Validation of the method

4.3.1. ***Accuracy:*** Accuracy was assessed using a minimum of three concentration levels in three replicate injections. In a preanalysed tablet solution having 80 µg/ml frusemide and 10 µg/ml amiloride hydrochloride, the standard solution of frusemide (80, 120 & 160 µg/ml) and amiloride hydrochloride (10, 15 & 20 µg/ml) were added in triplicate. The mean % recovery was found to be 99.98% for frusemide and 100.22% for amiloride hydrochloride. The limit for mean recovery is 90-107 %. Thus, the method was found to be accurate. Table 2 represents the accuracy data obtained for the method.

4.3.2. ***Precision:*** Precision was measured in terms of injection repeatability of method developed. Injection repeatability was assessed using six determinations at 100 percent of the test concentration 80µg/ml and 10µg/ml of frusemide and amiloride Hydrochloride respectively. The injection repeatability study showed a relative standard deviation (RSD) of 0.069 % for frusemide and 0.400 % for amiloride Hydrochloride (≤ 2). Thus, it shows that the developed method is sensitive and has ability to detect small changes in the concentration of drugs in the given sample solutions. Therefore, it is concluded that the analytical technique showed a good repeatability precision. Table 3 represents the precision data obtained for the method.

4.3.3. Robustness: Robustness of the method was determined by analyzing same sample blend at normal operating conditions and also by changing some operating analytical conditions such as flow rate, pH of the solution. The parameters and results of normal operating conditions (original) against changed conditions are included in Table 4 and 5.

These data were subjected to ANOVA test to see any significant difference between the data sets. No significant ($p< 0.02$) difference in mean % assay was found as the calculated value of F is lower than the critical value of F. Hence, the robustness of the method is established to the extent of variations applied to the experimental conditions.

4.3.4. Analysis of the marketed formulation: The developed method was applied to the analysis of the frusemide and amiloride hydrochloride in the marketed tablet formulation Amifru (batch no. 0605004, Elder Pharmaceuticals). The results of analysis are given in Table 6 and 7. The contents of frusemide and amiloride hydrochloride were found in the range with RSD less than 2% which indicates the suitability of the method for routine analysis of frusemide and amiloride hydrochloride in pharmaceutical dosage forms.

4.4.**Stability indicating property:** The % drugs recovery, retention time of frusemide & amiloride hydrochloride (min) with stress conditions and time duration are given in Table 8. The stressed condition samples are evaluated relative to the control sample with respect to % drugs recovery, retention time. The high % degradation of frusemide & amiloride hydrochloride indicates that the drugs are susceptible to wet heat, dry heat, day light, acidic and alkaline conditions with maximum degradation observed in oxidation degradation. Dry heat < Wet heat < Acid < Alkaline < Day light < Oxidation.

5.Discussion:

It is evident from the study of RP- HPLC method that the newly developed method can be used for routine analysis as an alternate method for the simultaneous estimation of frusemide and amiloride Hydrochloride in bulk and tablet dosage form. It can also be employed for stability studies in the pharmaceutical industry 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:

The developed HPLC technique is precise, accurate and stability indicating. Statistical analysis proves that the method is reproducible and selective for the analysis of Frusemide and Amiloride hydrochloride in pharmaceutical tablet dosage form. As the method estimates the tablet dosage form in presence of their degradation products, it can be employed as a stability indicating method.

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Figure 1: Chromatogram of frusemide at retention time of 3.040 min.

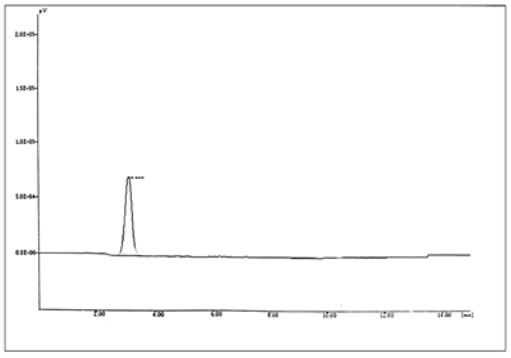

Figure 2: Chromatogram of amiloride hydrochloride at retention time of 10.004 min.

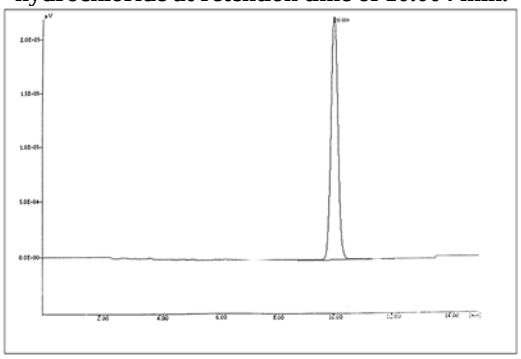

Figure 3: Chromatogram of frusemide and amiloride hydrochloride at retention time of 3.038 and 10.002 min. respectively.

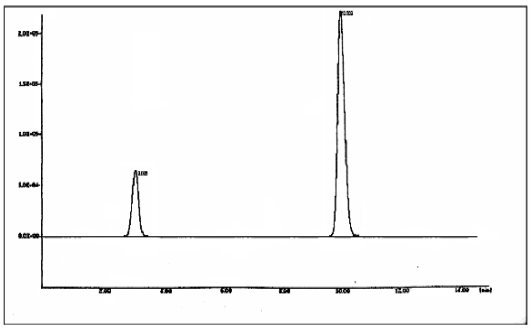

Table 1: System suitability parameters

Parameters	Acceptance Criteria	Observation of Frusemide	Observation of Amiloride HCl
Tailing factor	T ≤ 2	1.04	1.01
Theoretical plate	N > 2000	2979	9900
Asymmetry	As ≤ 2	1.01	1.00
Retention time	-----	3.040 min	10.004 min
Resolution	Rs > 2	6.854	

Table 2: Accuracy of frusemide and amiloride hydrochloride in Amifru™ tablet

Conc. before spiking (µg/ml)	Reference Std. added* (µg/ml)	Conc. after spiking* (µg/ml)	% Recovery
79.78	80	159.57	99.74
9.66	10	19.65	100.10
79.78	120	199.76	99.98
9.66	15	24.77	100.73
79.78	160	239.64	99.91
9.66	20	29.62	99.82
Mean ± SD# Fru		99.98 ± 0.123	
Mean ± SD# Ami		100.22 ± 0.466	

***** in triplicate

Table 3: Precision analysis of the method (Injection repeatability analysis)

Conc. (µg/ml)	Peak Area (µV*sec)	Mean ± SD	% RSD†
Fru + Ami (80 +10) µg/ml	695942 116494 694964 116491 695955 116541 696162 116380 696366 117230 695874 117355	**Fru** 695877.20 ± 482.542 **Ami** 116748.50 ± 426.52	**Fru** 0.069 **Ami** 0.400

† calculated from (SD/Mean*100)

Table 4: Robustness of method (Change in the flow rate)

Inj.	Flow rate ml/min	Ret. time Fru (min)	Ret. time Ami(min)	P. Area Fru (µV*sec)	P. Area Ami (µV*sec)
A	1.0	3.040	10.004	695942	116494
B	0.9	4.028	11.066	695564	115404
C	1.1	2.684	9.890	697308	117302
mean±SD				696271.33 ±	116400 ±
% RSD				918.00	952.48
				0.13	0.818

Table 5: Robustness of method (Change in the pH)

Inj.	pH	Ret. time Fru (min)	Ret. time Ami(min)	P. Area Fru (µV*sec)	P. Area Ami (µV*sec)
A	3.0	3.040	10.004	695942	116494
B	2.7	3.125	10.022	695542	115802
C	3.3	3.050	10.010	697277	116300
mean				696253.66 ±	116198.7 ±
±SD				908.51	356.95
%				0.130	0.307
RSD¶					

¶calculated from (SD/Mean*100)

Table 6: Analysis of marketed formulations in AMIFRU ™ tablets

Peak Area (µV*sec)		Label Qty. (mg/tab)		Quantity found (mg/tab)		% of Drug	
Fru	**Ami**	**Fru**	**Ami**	**Fru**	**Ami**	**Fru**	**Ami**
696535	117712	40	5	39.73	4.80	99.33	95.70
696374	117233			39.90	4.80	99.73	95.53
696651	117732			39.91	4.74	99.77	94.84
Mean				39.85	4.78	99.61	95.35

Table 7: Statistical analysis of AMIFRU ™

Parameters	Frusemide	Amiloride hydrochloride
% Conc. ±SD	99.61 ± 0.243	95.35 ±
% Coefficient of Variation	0.244	0.455
Standard Error Mean (SEM)	0.140	0.477
Percentage Range of Error	0.274	0.262
(within 95 % confidence limits)		0.515

Table 8: Specific stability study

Conditions	Time Duration (hours)	(%) Drugs recovery of Frusemide & Amiloride hydrochloride	Retention Time of Frusemide & Amiloride hydrochloride (min)
Normal solution	-	99.86, 99.74	3.038, 10.002
Wet heat	1	97.65, 97.50	3.046, 10.013
Dry heat	1	98.88, 98.90	3.041, 10.008
Day light	17	91.82, 89.50	3.070, 10.038
Oxidation,H_2O_2	6	88.54, 84.20	3.085, 10.053
Acidic	8	96.78, 97.40	3.051, 10.018
Alkaline	8	96.65, 96.50	3.053, 10.016