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Research Article

# SIMULTANEOUS ESTIMATION AND FORCED DEGRADATION STUDIES OF AMILORIDE HYDROCHLORIDE AND FUROSEMIDE IN A PHARMACEUTICAL DOSAGE FORM USING REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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**ABSTRACT**

**Objective**: The present study describes the stability indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous estimation of amiloride hydrochloride and furosemide in pharmaceutical dosage forms.

**Methods**: The proposed RP-HPLC method was developed using Shimadzu LC-2030 HPLC system equipped with UV detector, and chromatographic separation was carried on Shim-pack C18 (250 mm×4.6 mm, 5 µ) column at a flow rate of 1 ml/min and the runtime was 4min. The mobile phase consisted of water and acetonitrile in the ratio of 35:65, and elements were scanned using a UV detector at 281 nm.

**Results**: The retention time of amiloride hydrochloride and furosemide was found to be 1.92 min and 3.14min, respectively. Linearity was found to be 12–28 ppm for amiloride hydrochloride and 96–224 ppm for furosemide, respectively. Limit of detection and limit of quantification for amiloride hydrochloride were 0.381 ppm and 1.156 ppm and for furosemide were 2.00 ppm and 6.068 ppm, respectively.

**Conclusion**: The stability indicating method was developed by subjecting the drugs to stress conditions such as acid and base hydrolysis, oxidation, humidity, photolytic, and thermal degradation, and the degraded products formed were resolved successfully from the samples.

**Keywords:** Amiloride hydrochloride, Furosemide, Reverse-phase high-performance liquid chromatography, Degradation, Validation, ICH.

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## INTRODUCTION

Amiloride hydrochloride is a potassium-sparing diuretic. It is chemically 3,5-diamino-N-(diaminomethylene)-6-chloropyrazinecarboxami demonohydr ochloridedihydrate [1,2] (Fig. 1). It works by inhibiting sodium reabsorption in renal epithelial cells by binding to sodium channels. Inhibition of sodium reabsorption creates a negative voltage in the luminal membranes of principal cells, situated at the distal convoluted tubule and collecting duct. This negative voltage decreases the potassium and hydrogen ion secretion [2,3]. It is used in conjunction with diuretics to spare potassium loss.

Furosemide is a loop diuretic. It is chemically 4-chloro-2- furfurylamino-5-sulphamoyl benzoic acid (Fig. 1). Furosemide inhibits water reabsorption in the nephron by blocking sodium and chloride reabsorption in the ascending limb of the loop of Henle [4]. Furosemide helps to maintain potassium and minimize the risk of alkalosis, in the treatment of edema associated with hepatic cirrhosis and congestive heart failure [2,5].

The thorough literature survey reveals that few analytical methods such as RP-HPLC and UV methods are reported for simultaneous estimation of amiloride hydrochloride and furosemide in pharmaceutical dosage forms [3,6,7]. Thus, the present investigation was held out to acquire new, simple, accurate, rapid, and cost-effective stability indicating RP-HPLC method for the simultaneous estimation of amiloride hydrochloride and furosemide in pharmaceutical dosage form. The suggested method was applied successfully to split up the degraded products from the samples.

## METHODS

### Reagents and chemicals

Amiloride hydrochloride and furosemide standards were provided by Alkem Laboratories, Navi Mumbai, Maharashtra, India, and Yarrow Chem Products, Dombivili, Maharashtra, India. Commercial tablet dosage form, Frumil, was purchased from local markets. The HPLC grade acetonitrile and water were purchased from Thomas Baker. Analytical grade orthophosphoric acid (OPA), hydrochloric acid, sodium hydroxide, and hydrogen peroxide were purchased from S.D. Fine Chemicals.

### Instrument

The chromatographic separation was carried out by Shimadzu LC-2030 HPLC system equipped with UV detector and autosampler. The LabSolution software was used for signal monitoring and processing. Photolytic degradation was done in UV chamber, and hot air oven was employed for thermal degradation.

### Selection of wavelength

Both the drugs were scanned by UV individually, in a wavelength range of 200–400 nm and maxima for each drug was measured. The corresponding UV spectrum graphs of the drugs such as amiloride hydrochloride and hydrochlorothiazide are shown in Fig. 2. The detection wavelength was selected from the overlay UV spectrum and was found to be 281 nm.

### Chromatographic conditions

The chromatographic separation of analytes was carried out using ShimadzuRP-HPLC system with Shim-pack GIST C18 (250 × 4.6 mm, 5 µ) column. The mobile phase of a mixture of water and acetonitrile was in the ratio of 35:65 and column temperature was maintained at 25°C. The analytes were detected at 281 nm using UV detector. The runtime was set at 4 min at a flow rate of 1 ml/min.

### Preparation of standard stock solution

Standard stock solutions of amiloride hydrochloride and furosemide were prepared separately by dissolving 100 mg of amiloride hydrochloride and 100 mg of furosemide in 100 ml volumetric flasks

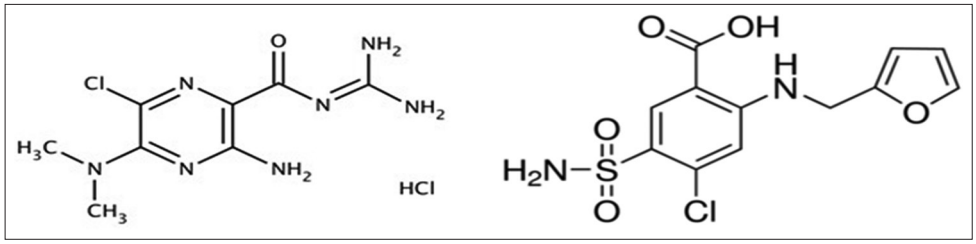


**Fig. 1: Structure of amiloride hydrochloride and furosemide**

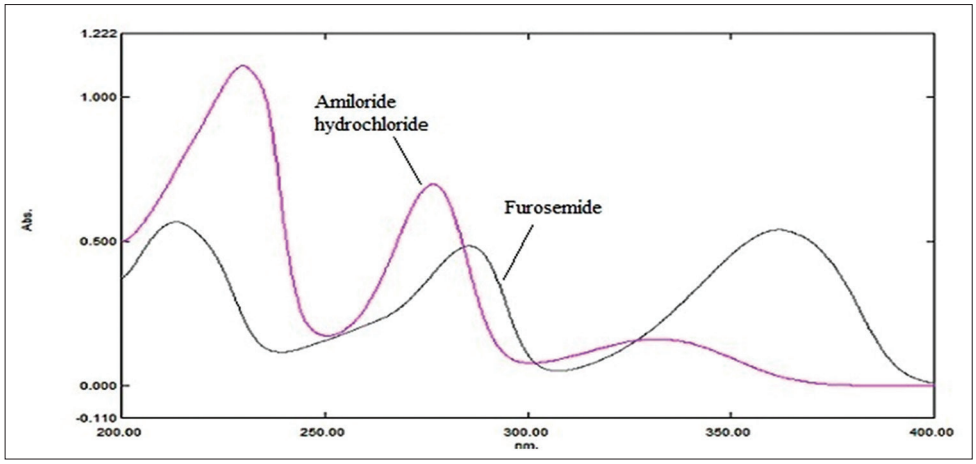


**Fig. 2: Overlain spectra of amiloride hydrochloride and furosemide**

with water:acetonitrile (35:65) as diluent and sonicated for 10 min. From the above solution, 0.2 ml of amiloride hydrochloride and 1.6 ml of furosemide were transferred separately to 10 ml volumetric flasks, and 1 ml 5% OPA was added as a supporter and sonicated for 5 min and made up the volume with diluent to get 20ppm of amiloride hydrochloride and 160ppm of furosemide standard stock solution.

**Preparation of sample solution**

Ten tablets (Frumil tablets: 5 mg amiloride hydrochloride and 40 mg furosemide) were weighed and the average weight of each tablet was calculated; then, the weight equivalent to 1 tablet was transferred into a 100 ml clean dry volumetric flask, add 30 ml of diluent, sonicated for 25 min, and make up to the final volume with diluent and filtered. 2 ml of the filtered solution was pipetted out into a 10 ml volumetric flask and 1 ml 5% of the OPA was added as a supporter and sonicated for 5 min, and volume made up to 10 ml with diluent.

**Method validation**

The method validation was done according to the ICH guidelines with above developed RP-HPLC method for simultaneous estimation of amiloride hydrochloride and furosemide. Several parameters were evaluated such as system suitability, precision, accuracy, linearity, robustness, limit of detection (LOD), and limit of quantification (LOQ) [8,9].

**Forced degradation studies**

The ICH degradation was attempted under various stress conditions such as acid, alkaline, oxidation, thermal, humidity, and photolytic conditions to evaluate the interference of degradation impurities. These studies help to know the inherent stability characteristic of the active molecules in drug product and the possible degradation products.

Acid, base, and oxidation degradations were performed by adding 5 ml of 1 N HCl, 5 ml of 1 N NaOH, and 5 ml of 30% peroxide solution ($H_2O_2$), respectively, to the sample solutions, and these samples were kept at room temperature for 3 h. Thermal degradation was performed by keeping the tablets in a Petri dish and then placed them in an oven at 105°C for 48 h. Humidity degradation was performed by placing the tablets in a Petri dish and kept in a humidity chamber at 95% relative humidity, at 25°C for 120 h. A photolytic degradation study was carried out by placing the tablets in a Petri dish in a photolytic chamber for 7 days.

## RESULTS AND DISCUSSION

**Method development**

A series of tests was taken with different columns such as Inertsil ODS and Shim-pack C18 column with different mobile phases to produce a suitable RP-HPLC method for estimation of amiloride hydrochloride and furosemide in tablet dosage form, and finally, a typical chromatogram was obtained with water and acetonitrile in the ratio of 35:65. The chromatographic separation was performed on Shim-Pack C18 (250 × 4.6 mm, 5 µ) column by injecting 20 µL, and the analytes were detected with UV detector at 281 nm. The retention time of amiloride hydrochloride and furosemide was found to be 1.92 min and 3.14 min, respectively. Forced degradation studies for amiloride hydrochloride and hydrochlorothiazide in tablet dosage form were also carried out using the developed method, and the degraded compounds were effectively resolved. The optimized conditions were given in Table 1 and Fig. 3.

**System suitability**

System suitability was performed to verify the acceptability of the resolution and repeatability of the system. System suitability was performed by injecting six replicate injections of the standard solution (100%), and parameters such as peak area, USP tailing, theoretical plates, retention time, and peak asymmetry were evaluated. The % relative standard deviation (RSD) was determined and reported within the limits [10]. The results are shown in Table 2.

**Accuracy**

The accuracy of the proposed method was evaluated by calculating the recovery studies of the test drug at three different concentration levels (80%, 100%, and 120%) by the standard addition method [11]. A known amount of amiloride hydrochloride and furosemide was added to the prequantified sample solution, and three replicates of each concentration

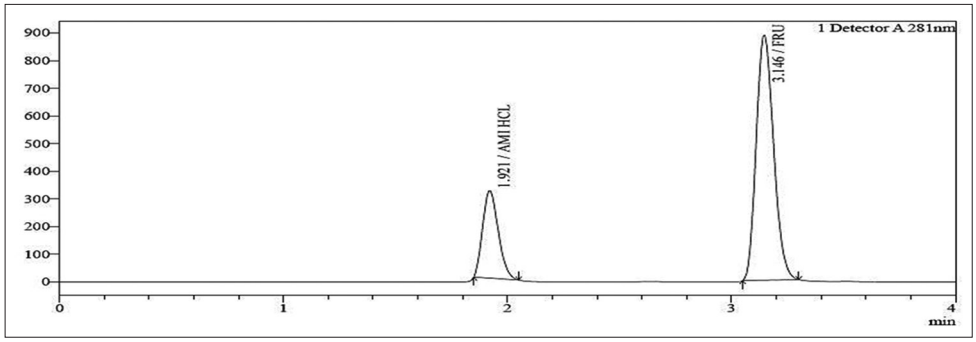


**Fig. 3: Chromatogram of standard amiloride hydrochloride and furosemide**

**Table 1: Optimized chromatographic condition**

| Parameter | Optimized condition |
|---|---|
| Column | Shim-Pack C18 (250×4.6 mm, 5 µ) column |
| Mobile phase | Water and acetonitrile in the ratio of 35:65 |
| Flow rate | 1 ml/min |
| Wavelength | UV detector at 281 nm |
| Injection volume | 20 µL |
| Temperature | 25°C |
| Retention time | Amiloride hydrochloride 1.921 min and furosemide 3.146 min |

**Table 2: System suitability parameters**

| Parameters | Amiloride hydrochloride | Furosemide |
|---|---|---|
| Retention time (min) | 1.921 | 3.146 |
| USP plate count | 2876 | 6454 |
| USP tailing | 1.243 | 1.191 |

were injected into developing chromatographic conditions. The percentage recovery result of amiloride hydrochloride and furosemide was found to be within limits of 100–101%, indicating that the developed method was found to be accurate. The percentage recovery results are shown in Table 3.

**Precision**

The precision of an analytical procedure may be defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The method precision and system precision studies were carried out by injecting six replicates of both standard and test solutions with the same concentration [12]. The % RSD was calculated from the chromatogram and the results obtained were within the limits of 2%, and the proposed method was found to be precise. The precision data are given in Table 4.

**Linearity**

The linearity of the method was determined at different concentration levels ranging from 12 to 28 ppm of amiloride hydrochloride and from 96 to 224 ppm of furosemide. All the concentrations were prepared and injected into the system. The linearity curve was constructed by plotting peak area versus concentration of the analyte. From the results obtained, the proposed method was found to be linear. The regression coefficient ($r^2$) was found to be 0.999 and 0.999 for amiloride hydrochloride and furosemide, respectively, and the result is shown in Fig. 4.

**LOD and LOQ**

In the present study, the LOD and LOQ of amiloride hydrochloride and furosemide were evaluated based on the standard calibration curve method [13]. LOD is performed to know the lowest concentration level of the analyte that gives a measurable response. LOD and LOQ for amiloride hydrochloride are 0.381 ppm and 1.156 ppm and for furosemide are 2.00 ppm and 6.068 ppm, respectively.

**Robustness**

Robustness of the proposed method has been evaluated by small deliberate changes in the system parameters such as flow rate, wavelength, and temperature [14]. It was found that none of the above parameters caused an alteration in the peak area, retention time, and USP tailing by small changes such as ±0.2 ml change in flow rate, ±2 nm wavelength, and ±2°C changes in temperature. The % RSD was found to be within the limits, and the method was found to be robust. The robustness results are shown in Table 5.

**Assay of marketed formulation**

Analysis of marketed formulation (Frumil tablets: 5 mg amiloride hydrochloride and 40 mg furosemide) was purchased from local markets. Ten tablets were weighed and average weight of each tablet was calculated; then, the weight equivalent to 1 tablet was transferred into a 100 ml clean dry volumetric flask, add 30 ml of diluent, sonicated for 25 min, and make up to the final volume with diluent and filtered. 2 ml of the filtered solution was pipetted out into a 10 ml volumetric flask, and 1 ml 5% of OPA is added as a supporter and made up to 10 ml with diluent. From the resulting solution, 20 µL was injected into HPLC system and peak areas were recorded. The % assay of the marketed formulation was found to be 99.55% for amiloride hydrochloride and 99.9% for furosemide as shown in Table 6.

**Forced degradation studies**

Forced degradation studies of the drug formulation were carried out by treating the drug samples under stress-induced conditions such as acid and base hydrolysis, oxidation, humidity, and photo- and thermal-degradation to evaluate the ability of the proposed method to separate amiloride hydrochloride and furosemide from its degradation products as shown in Figs. 5-11. The % assay of amiloride hydrochloride and furosemide with respect to untreated sample and % assay results obtained from treating the samples with various stress conditions had a difference which was within the acceptable limits. The results of stress studies are shown in Table 7.

**Acid degradation**

Acid (1 N hydrochloric acid) degradation study showed 1.3% and 1.9% degradation for amiloride hydrochloride and furosemide, respectively. However, no degradation product was observed in the chromatogram (Fig. 6).

**Alkali degradation**

The degradation in base (1 N sodium hydroxide) was found to be 1.1% and 3.9% for amiloride hydrochloride and furosemide, respectively. However, no degradation product was observed in the chromatogram (Fig. 7).

**Oxidative degradation**

Oxidative degradation study in 30% hydrogen peroxide gave around 2.2% and 6.9% for amiloride hydrochloride and furosemide, respectively. However, no degradation product was observed in the chromatogram (Fig. 8).

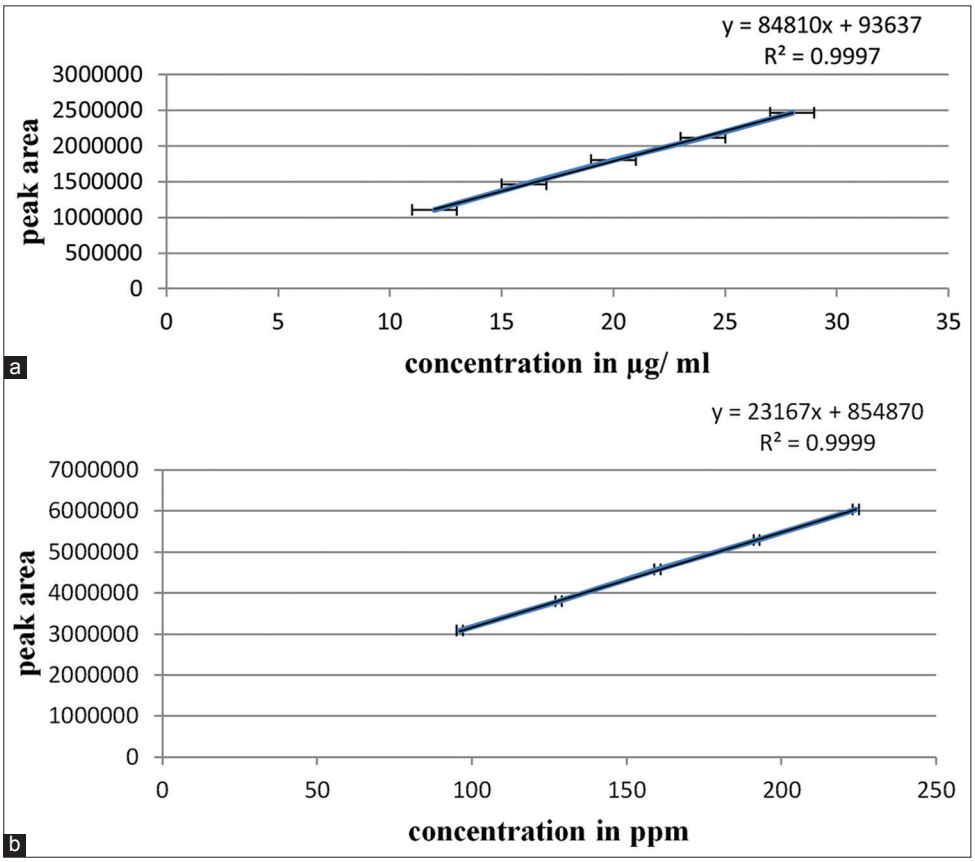


**Fig. 4: Linearity graph of (a) amiloride hydrochloride and (b) furosemide**

**Table 3: Percentage recovery results of amiloride hydrochloride and furosemide**

| Spiked (%) | Percentage recovery | | Mean percentage recovery | %RSD | |
|---|---|---|---|---|---|
| | Amiloride hydrochloride | Furosemide | | Amiloride hydrochloride | Furosemide |
| 80 | 99.7 | 100 | 100.1 | 0.74 | 0.50 |
| | 100.8 | 99.6 | | | |
| | 101.1 | 100.6 | | | |
| 100 | 100.2 | 100 | 100.1 | 0.47 | 0.06 |
| | 100 | 99.9 | | | |
| | 100.9 | 100 | | | |
| 120 | 101 | 99.7 | 100.7 | 0.23 | 0.61 |
| | 101.4 | 100.7 | | | |
| | 101 | 100.8 | | | |

RSD: Relative standard deviation

**Table 4: Results of method precision for amiloride hydrochloride and furosemide**

| | % Assay | |
|---|---|---|
| S. No. | Amiloride hydrochloride | Furosemide |
| 1 | 101.1 | 100.9 |
| 2 | 101.7 | 100.2 |
| 3 | 101 | 100.6 |
| 4 | 101.6 | 100.5 |
| 5 | 100.9 | 99.7 |
| 6 | 101.5 | 100.2 |
| Mean±SD | ±0.35 | ±0.41 |
| %RSD | 0.35 | 0.41 |

SD: Standard deviation, RSD: Relative standard deviation

**Photolytic degradation**

In photolytic UV degradation, the drug degraded was 0.3% and 0.65% for amiloride hydrochloride and furosemide, respectively. However, no degradation product was observed in the chromatogram (Fig. 9).

**Thermal degradation**

In thermal degradation, there was no degradation peak observed in the chromatogram, and degradation was 1.2% and 2.7% for amiloride hydrochloride and furosemide, respectively (Fig. 10).

**Humidity degradation**

In humidity degradation, the drug degraded was 0.6% and 11.5% for amiloride hydrochloride and furosemide, respectively. However, no degradation product was observed in the chromatogram (Fig. 11).

**Table 5: Results of robustness**

| S. No. | Parameter | Amiloride hydrochloride | | | Furosemide | | |
|---|---|---|---|---|---|---|---|
| | | RT | NTP | TF | RT | NTP | TF |
| 1 | Flow rate 0.8 ml | 2.313 | 3357 | 1.220 | 3.663 | 8027 | 1.178 |
| | Flow rate 1.2 ml | 1.643 | 2397 | 1.270 | 2.531 | 5872 | 1.197 |
| 2 | Temperature 23°C | 1.927 | 2881 | 1.249 | 3.158 | 6488 | 1.193 |
| | Temperature 27°C | 1.922 | 2896 | 1.240 | 3.147 | 6511 | 1.192 |
| 3 | Wavelength 279 nm | 1.923 | 2846 | 1.237 | 3.141 | 6337 | 1.196 |
| | Wavelength 283 nm | 1.921 | 2885 | 1.239 | 3.145 | 6721 | 1.192 |

RT: Retention time, NTP: Number of theoretical plates, TF: Tailing factor

**Table 6: Percentage content of marketed formulation**

| Tablet | Drug | Amount taken | Amount found | % Assay |
|---|---|---|---|---|
| FRUMIL (amiloride hydrochloride 5 mg and furosemide 40 mg) | Amiloride hydrochloride | 20 ppm | 19.91 ppm | 99.55 |
| | Furosemide | 160 ppm | 159.84 ppm | 99.90 |

**Table 7: Forced degradation studies of amiloride hydrochloride and furosemide**

| Stress condition | Amiloride hydrochloride | | Furosemide | |
|---|---|---|---|---|
| | % Assay | % Difference w.r.t control | % Assay | % Difference w.r.t control |
| Control | 99.7 | NA | 99.8 | NA |
| Acid degradation | 98.4 | 1.3 | 97.9 | 1.9 |
| Base degradation | 98.6 | 1.1 | 95.9 | 3.9 |
| Oxidative degradation | 97.5 | 2.2 | 92.9 | 6.9 |
| Photolytic degradation | 99.4 | 0.3 | 99.3 | 0.5 |
| Thermal degradation | 98.5 | 1.2 | 97.1 | 2.7 |
| Humidity degradation | 99.1 | 0.6 | 88.3 | 11.5 |

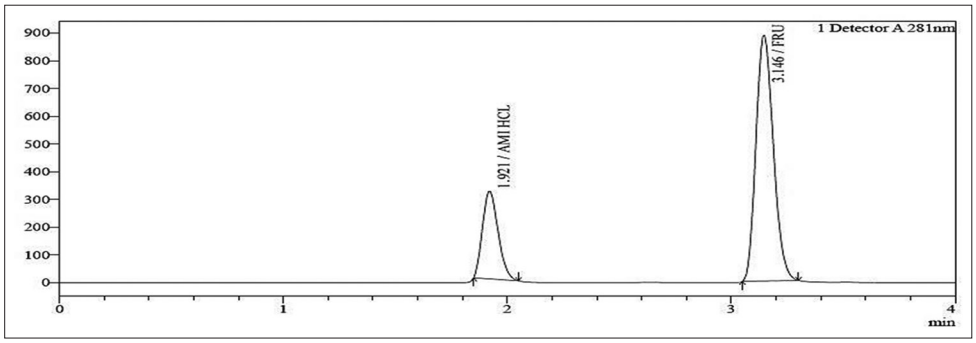


**Fig. 5: Chromatogram of untreated tablet**

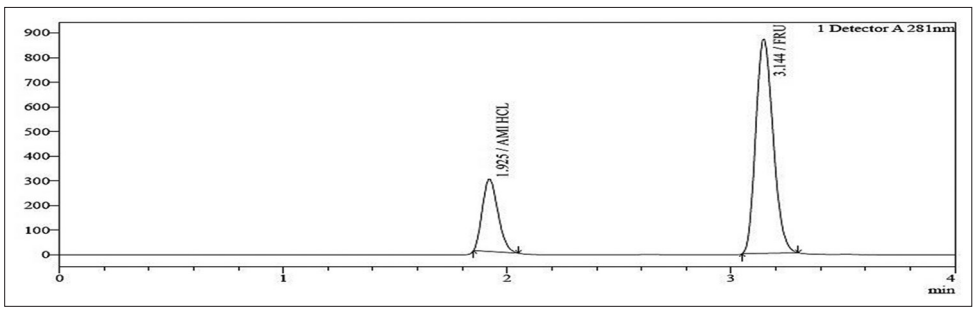


**Fig. 6: Chromatogram of acid degradation**

## CONCLUSION

Stability indicating RP-HPLC method has been developed and for simultaneous estimation of amiloride hydrochloride and furosemide in tablet dosage form. The validated method was successfully implemented for the stress testing and analysis of amiloride hydrochloride and furosemide. The stress testing studies revealed that the method was successfully employed to resolve the degraded products from the sample. The proposed method was proved to be selective, accurate, precise, and rapid, and it can be used for the routine analysis of the amiloride hydrochloride and furosemide in the formulation.

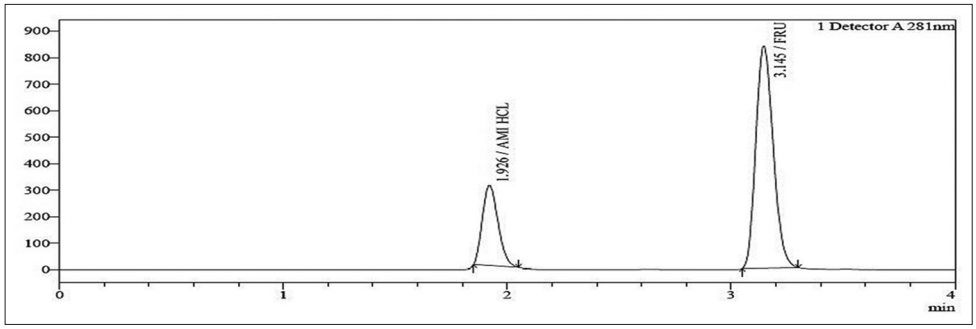


**Fig. 7: Chromatogram of base degradation**

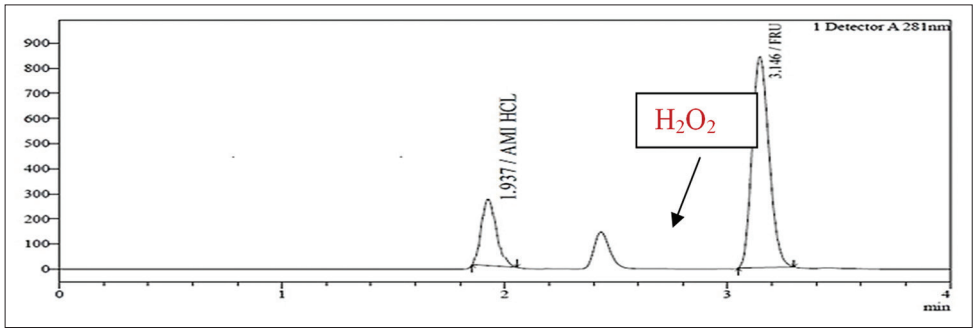


**Fig. 8: Chromatogram of oxidative degradation**

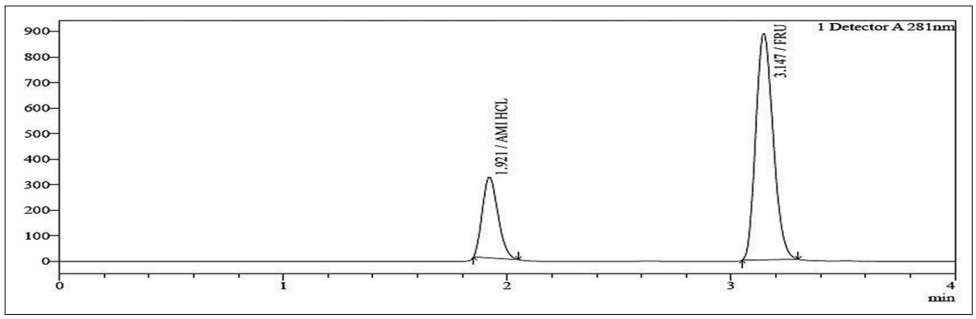


**Fig. 9: Chromatogram of photolytic degradation**

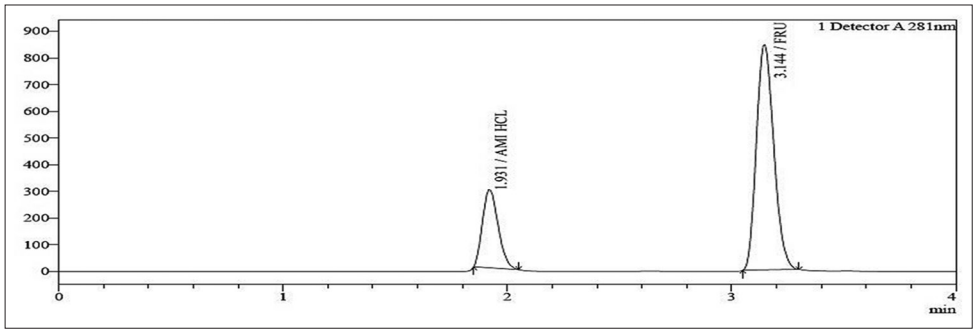


**Fig. 10: Chromatogram of thermal degradation**

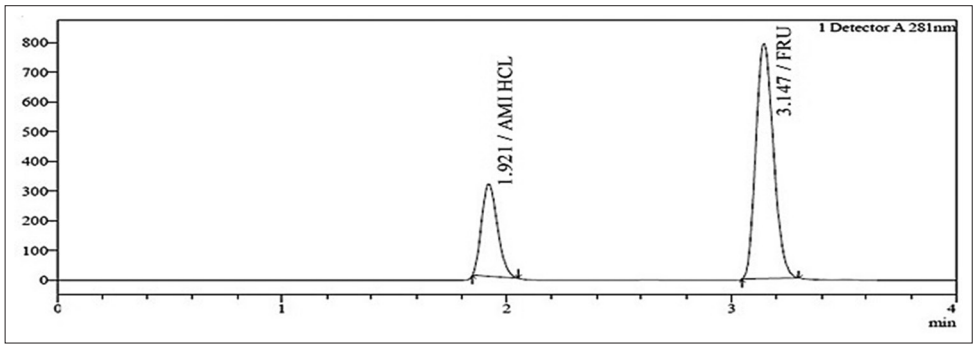


**Fig.11: Chromatogram of humidity degradation**

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**AUTHORS' CONTRIBUTION**

All the authors have contributed equally.

**CONFLICTS OF INTEREST**

The authors confirm that this paper has no conflict of interests.

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