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STRESS DEGRADATION STUDIES OF FUROSEMIDE AND DEVELOPMENT AND VALIDATION OF SIAM RP-HPLC METHOD FOR ITS QUANTIFICATION

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ABSTRACT

Stress degradation studies have been carried out on Furosemide and novel SIAM RP-HPLC method has been developed and validated for quantitative analysis of Furosemide. Use of a Supelcosil C8 (250 mm × 4.6 mm, 5-µm particle size) column. The mobile phase was 70:30 (*v*/*v*) of 0.2 g of potassium dihydrogen orthophosphate and 0.25 g of cetrimide in 70 mL of water adjust the pH to 7.0 with 6M ammonia and add 30 mL of 1-propanol as isocratic mobile phase enabled separation of the drug from its degradation products. The flow rate and detection wavelength were 1 ml/min and 238 nm respectively. The method was validated for linearity, limits of detection and quantification, accuracy, precision and robustness. The linearity of the method was excellent over the range 16–24µg/ml. RSD in intra-day and inter-day precision studies was < 2%. Recovery of Furosemide from bulk drug ranged from 99.59 and 102.33%. Furosemide was subjected to stress conditions (hydrolysis (acid, base), oxidation, photolysis, and thermal degradation) and the stressed samples were analyzed by use of the method. The degradation products were the related impurities and were well resolved from main peak thus proving the stability indicating nature of the method.

KEYWORDS: Furosemide; Stress degradation; SIAM; related impurities.

INTRODUCTION

Furosemide, is 5-(Aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)-amino]-benzoic acid dihydropyridine-3,5-dicarboxylate. Furosemide is a loop diuretic acting as antihypertensive

agent.[1-2] The exact mode of action of Furosemide is defined precisely in comparison to ethacrynic acid which does not bind sulfhydryl groups of renal cellular proteins. Inhibition of reabsorption of electrolytes by Furosemide in the ascending limb of loop of Henle is an important mechanism of action. The drug is found to decreases re-absorption of chloride and sodium. In addition, potassium excretion in distal renal tubule is increased which exerts direct effect on the transport of electrolytes at proximal tubule. Carbonic anhydrase inhibition is not observed with furosemide; also it is not an aldosterone antagonist. Inhibition of coupled Na+/K+/2Cl- transport system in the luminal membrane of loop of Henle (thick ascending limb), is been reported with furosemide. Hence it is said that loop diuretics tend to reduce the re-absorption of sodium chloride and also reduce the normal lumen-positive potential derived from K+ recycling. Furosemide is advised for treating edema associated with cirrhosis of the liver, congestive heart failure and renal diseases which include hypertension (single or in conjunction with other hypotensive agents) and nephritic syndrome.[3-5] The reported dose of Furosemide for edema is 20-40 mg every 8 hours and for hypertension the total dose is 80 mg, usually divided into 40 mg twice a day. For the treatment of edema 60 mg dose recommended as sustained release to reduce the high peak diuresis effect associated with conventional dosage. For oedematous conditions 60 mg daily is the recommended initial dose. In mild cases 60 mg on alternative days may be sufficient.[6-7]

Stability-indicating assay methods (SIAM's) can be specific one, which evaluate the drug in the presence of its degradation products, excipients and additives, or selective one which is able to measure the drug and all the degradation products in the presence of excipients and additives. The International Conference on Harmonization (ICH) guidelines requires performing stress testing of the drug substance, which can help identify the likely degradation products. Moreover, validated stability-indicating method should be applied in the stability study. Stability is considered as one of the most important criteria in pharmaceutical quality control. Only stable preparation would promise precise delivery of the drug to the patients. Expiration dating on any drug product is based upon scientific studies at normal and stressed conditions.[8]

Hence an attempt has been made to develop and validate a sensitive stability indicating high performance liquid chromatographic method for Furosemide which is specific, precise and accurate.

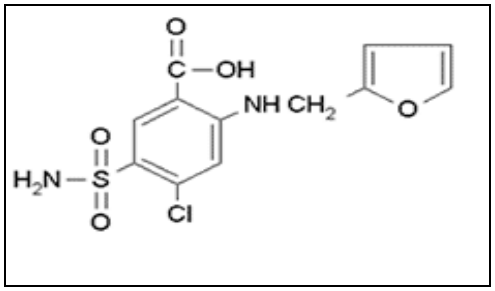


Figure: 1. Structure of Furosemide

MATERIALS AND METHOD

Reagents and Materials

HPLC grade 1-propanol was purchased from Merck (Darmstadt, Germany). Deionised and ultra pure water used in all experiments was obtained from Milli-Q System (Millipore). The 0.45-µm Nylon pump filter was obtained from Advanced Microdevices (Mumbai, India). Orthophosphoric acid used for adjusting the pH of buffer solution was of AR grade (Merck, Darmstadt, Germany). Potassium dihydrogen orthophosphate (KH_2PO_4), ammonia (NH3), sodium hydroxide (NaOH), cetrimide, hydrochloric acid (HCl), and hydrogen peroxide (H_2O_2) were purchased from Qualigens Fine Chemicals (Mumbai, India).

Preparation of Mobile Phase

0.2 g of potassium dihydrogen orthophosphate and 0.25 g of cetrimide was added in 70mL of water, the pH was adjusted to 7.0 with 6M ammonia and 30 mL of 1-propanol was added.

Preparation of Stock Solution

Furosemide working standard (20 mg) was weighed accurately and was transferred to 50 mL volumetric flask. About 40 mL mobile phase was added to the flask and solution was sonicated for 15 minutes. The solution was allowed to cool at room temperature and volume was made up to 50 mL with the mobile phase to give 400µg/mL solution of furosemide.

Preparation of Standard Solution

Stock solution (1mL) was pippetted out with the pipette into a 20mL volumetric flask and the volume made up to 20 mL with mobile phase. The solution was mixed to give of concentration 20µg/mL.

Chromatographic conditions

A Jasco Liquid Chromatographic system with PDA Detector was used for the method development and forced degradation studies. The chromatographic separation was achieved

on Supelcosil C8 (250 mm × 4.6 mm, 5-µm particle size) column, 70:30 (*v*/*v*) as mobile phase mentioned pumped at a flow rate of 1.0 ml min^{-1}. Before use it was filtered through a 0.45-µm Nylon filter and degassed in an ultrasonic bath. Analysis was carried out at room temperature. The injection volume was 100µL and ultraviolet (UV) detection was at 238 nm.

Stress Degradation Studies[9]

Stress testing helps us to elucidate the intrinsic stability characteristics of the drug molecule. It provides data on properties such as degradation pathways, leads to identification of degradation products and helps in developing the stability indicating HPLC assay method. Furosemide was subjected to various stress conditions as mentioned below.

- **Acid hydrolysis**

The drug was subjected to forced degradation under acidic condition (2N HCl). Furosemide (20 mg) was weighed accurately and was transferred to 50 mL volumetric flask containing 20 mL mobile phase. Hydrochloric acid solution of 2N (2mL) was added to flask and was heated at 70°C for 2 hrs in water bath. This solution was cooled to room temperature and was neutralized by using 2N NaOH solution to pH 7. Volume was made up to 50mL with mobile phase up to 50 mL to get 400 µg/mL solution of Furosemide. This solution was then injected to chromatography to obtain chromatogram.

- **Alkaline hydrolysis**

The drug was subjected to forced degradation under basic condition (2N NaOH). Furosemide (20 mg) was weighed accurately and was transferred to 50 mL volumetric flask containing 20 mL mobile phase. 2ml of NaOH solution (2N) was added in it and heated at 70°C for 2 hrs in water bath. This solution was cooled to room temperature and was neutralized by using 2N HCl solution to pH 7. Volume was made up to 50mL with mobile phase up to 50 mL to get 400µg/mL solution of Furosemide. This solution was then injected to chromatography to obtain chromatogram.

- **Oxidative degradation**

The drug was subjected to forced degradation under oxidation (15% v/v H2O2 solution). Furosemide (20 mg) was weighed accurately and was transferred to 50 mL volumetric flask containing 20mL mobile phase. 5mL of (15% v/v H2O2 solution) was added in it and heated it at 70°C for 10 minutes in water bath. This solution was neutralized with sodium

metabisulphate solution and volume was made up to 50 mL with mobile phase to get 400µg/mL solution of Furosemide. This solution was then injected to obtain chromatogram.

➢ **Photolytic degradation**

Furosemide (20 mg) was weighed accurately and was transferred to 50 mL volumetric flask containing 40 mL mobile phase. The solution was sonicated for 15 min and volume was made up to 50 mL with mobile phase. This solution containing 400µg/mL furosemide was subject to 1.2 million lux of white fluorescent light and 200 W of UV as per ICH guidelines. The Furosemide solution was analyzed for any signs of degradation at concentration of 400µg/mL.

➢ **Heat degradation**

The drug was subjected to dry heat degradation by exposing the powder sample of Furosemide to 80°C for 24 hrs in oven in closed condition. Sample solution was prepared to get final concentration to 400µg/mL of Furosemide in mobile phase. This solution was then injected to obtain chromatogram.

Method Validation

The method was validated for linearity, accuracy, precision, robustness, stability in accordance with ICH guidelines.

➢ **Limit of Detection**

The limit of detection was determined based on signal to noise ratio. The concentration, which gave a signal to noise ratio about 3.0, was derived for limit of detection. Solution of drug was injected six times at the LOD.

➢ **Limit of Quantification**

The limit of detection was determined based on signal to noise ratio. The concentration, which gave a signal to noise ratio about 10.0, was derived for limit of detection. Solution of drug was injected six times at the LOQ.

➢ **Linearity**

Linearity solutions was prepared by quantitative dilutions of the stock solution of furosemide reference standard to obtain solutions at 80%, 90%, 100%, 110%, and 120% of working concentration. Each solution was injected in triplicate and the mean area of the peak due to Furosemide was calculated. 1 mL of stock solution was diluted up to 10 mL with mobile

phase to give concentrations in the range from 16µg/mL, 18µg/mL, 20µg/mL, 22µg/mL 24µg/mL.

➢ **Accuracy**

To validate, that the test method can accurately quantify Furosemide within the formulation, three samples were prepared, each by spiking Furosemide raw material to equivalent amount of placebo at 80%, 100% and 120% of the working concentration (20µg/mL of Furosemide). Each level was weighed thrice and injected. Percentage Recovery (% Assay) for the drug was calculated for each level.

Level 80%

Furosemide 16 mg was added to 64 mg of placebo powder and subjected to the extraction in mobile phase in 50mL. During the final dilution stage, 1mL of this solution was pipetted into a 20 mL volumetric flask and diluted to 20 mL with mobile phase to give final concentration of 16µg/mL of Furosemide.

Level 100 %

Furosemide 20 mg was added to 64 mg of placebo powder and subjected to the extraction in mobile phase in 50mL. During the final dilution stage, 1mL of this solution was pipetted into a 20 mL volumetric flask and diluted to 20 mL with mobile phase to give final concentration of 20µg/mL of Furosemide.

Level 120 %

Furosemide 24 mg was added to 64 mg of placebo powder and subjected to the extraction in mobile phase in 50mL. During the final dilution stage, 1mL of this solution was pipetted into a 20 mL volumetric flask and diluted to 20 mL with mobile phase to give final concentration of 24µg/mL of Furosemide.

Percent recovery should be between 98% and 102% and the RSD of percent recovery of Furosemide should be less than 5%.

➢ **Precision**

Standard solution at working concentration 20µg/mL was injected six times. The RSD of the peak area of the six injections should not be more than 2%.

Repeatability

Standard solution at working concentration 20µg/mL was injected thrice.

Intra-day Precision

A standard drug solution of 20µg/mL in mobile phase from six different weighing was injected in triplicate. Relative standard deviation of the data gave a measure of accuracy of the method. The RSD of the area values the six injections should not be more than 2%.

Inter-day Precision

To demonstrate ruggedness of assay method, a variability test was conducted on the HPLC system by a different analyst on a different day as per test method. Standard solution at working concentration (20µg/mL) was injected three times. The procedure was repeated on Day 2 by Chemist 2. The RSD of the % area values of the injections should not be more than 2%. The difference in the area values on Day 1 should not be more than 3% from Day 2.

- **Robustness**

Change in flow rate

In order to study the robustness of system, the system suitability parameters were checked by injecting the standard and sample preparation with the flow rate of ± 0.2 mL/min viz. 0.8 mL/min and 1.2mL/min.

Change in mobile phase composition

To study the robustness, the system suitability parameters were checked by injecting the standard and sample preparation by changing the mobile phase composition by 10% viz. Buffer: 1-propanol, 67:33 and 73:27.

Change in mobile phase pH

In order to study the robustness, the system suitability parameters were checked by injecting the standard and sample preparation at mobile phase composition ± 0.2 units viz. with change in pH of mobile phase to pH 6.8 and pH 7.2 by using 6M ammonia solution.

Six injections of standard solution were injected to the chromatographic system and the chromatogram was recorded. Three injections of sample solution were injected to the chromatographic system and the chromatograms were recorded. The percent assay of each sample solution was calculated. The % relative standard deviation of peak area, retention

time of three standard solutions, the percent assay and retention times of two sample injections should not be more than 2%.

➢ **Solution stability**

The stability of furosemide sample solution and standard preparation were determined. The assay was performed on test preparation as per the test method. The standard solution as well as sample solution was kept at room temperature as well as in the fridge (Below 10 ºC) for 24 hrs and the sample solution and standard solution were injected at initial, after 12 hrs and after 24 hrs. Six injections of standard solution were injected to the chromatographic system and the chromatogram was recorded. Three injections of sample solution were injected to the chromatographic system and the chromatogram was recorded. The percent assay of each sample solution was calculated. The percent relative standard deviation of area and retention time of three standard solutions and the percent assay and retention times of two samples injections should not be more than 2%. The absolute difference in the % assay values of initial, 12 hrs and 24 hrs should not be more than 2%.

Assay of Furosemide tablets

Tablets (20) were randomly sampled and were crushed to a fine powder. Powder equivalent to 20 mg of Furosemide was weighed accurately and was transferred to 100 mL volumetric flask. About 80 mL of mobile phase was added and the solution was sonicated for 15 minutes. The volume was made up to 100 mL with mobile phase and solution was mixed. Solution was filtered through 0.45µ membrane filter, about 2mL of initial filtrate was discarded and remaining filtrate was collected. This solution (1 mL) was further diluted to 10 mL with mobile phase and was injected in to chromatography.

RESULTS AND DISCUSSION

Forced Degradation Studies

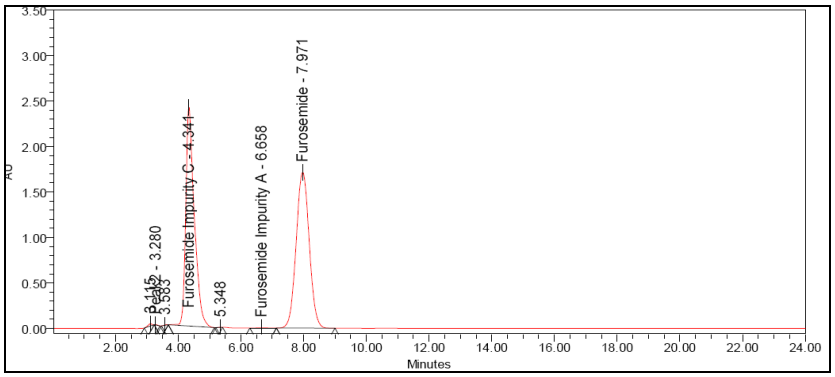


Figure: 2. Chromatogram of acid degradation of Furosemide

Acid degradation solution was prepared as per proposed method and the solution was injected to the chromatographic system. There was no degradation peak interfering with furosemide peak. The degraded peaks were well separated from furosemide peak.

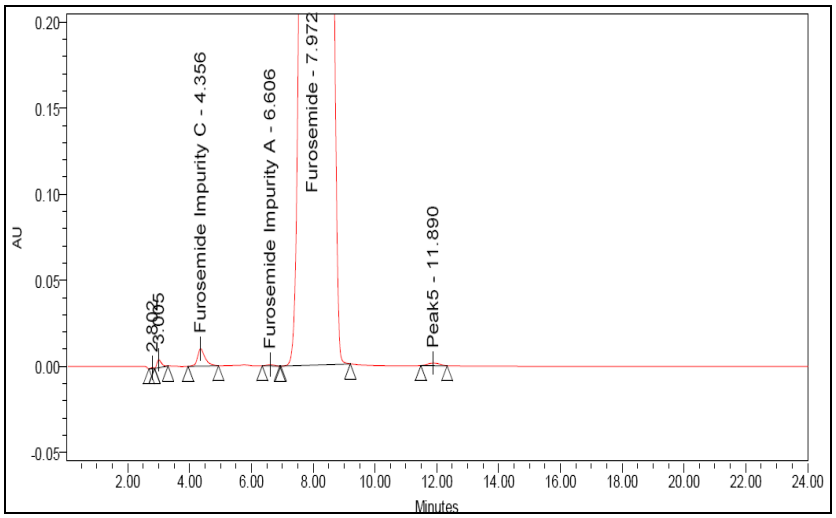


Figure: 3. Chromatogram of base degradation of Furosemide

Base degradation solution was prepared as per proposed method and the solution was injected to the chromatographic system. There was no degradation peak interfering with Furosemide peak. The degraded peaks were well separated from Furosemide peak.

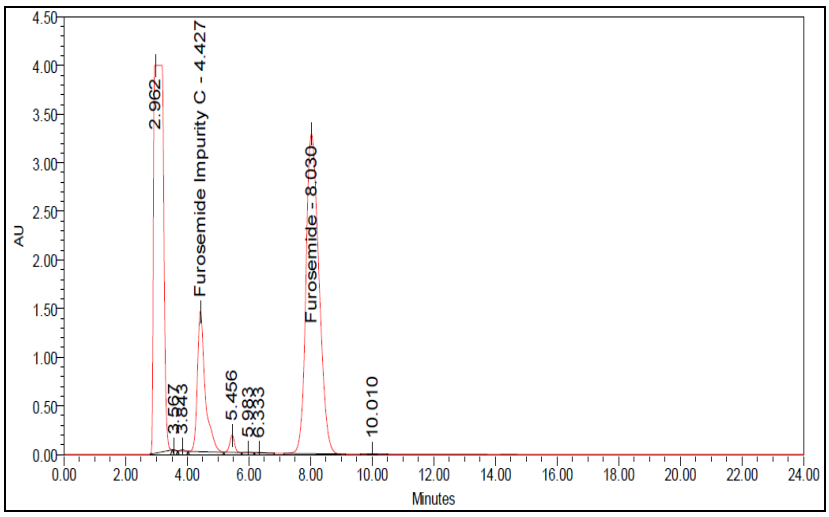


Figure: 4. Chromatogram of peroxide degradation of Furosemide

Peroxide degradation solution was prepared as per proposed method and the solution was injected to the chromatographic system. There was no degradation peak interfering with furosemide peak. The degraded peaks were well separated from furosemide peak.

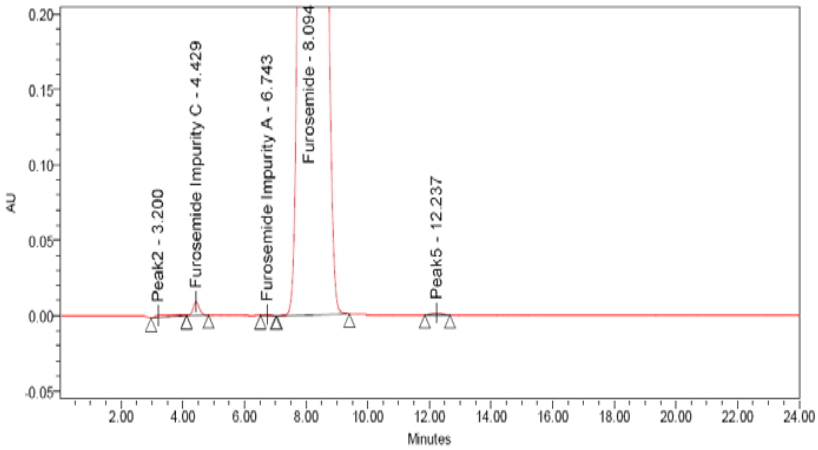


Figure: 5. Chromatogram of photo degradation of Furosemide

The Furosemide was exposed to UV light as per proposed method and the solution was injected to the chromatographic system. There was no degradation peak interfering with Furosemide peak. The degraded peaks were well separated from Furosemide peak.

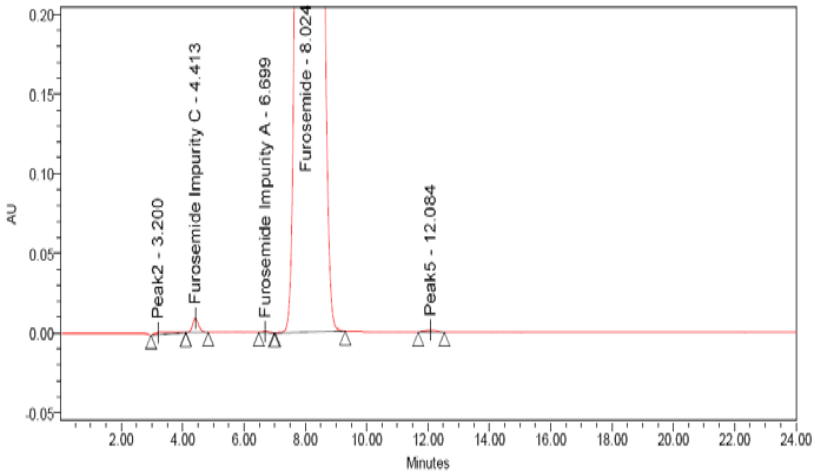


Figure: 6. Chromatogram of solid state heat degradation of Furosemide

The drug was subjected to dry heat degradation as per proposed method and the solution was injected to the chromatographic system. There was no degradation peak interfering with Furosemide peak. The degraded peaks were well separated from Furosemide peak.

Table 1: Retention time of Furosemide and its degradation product (Related Impurities)

Drug/Impurity	Retention Time (min)	Relative Retention Time
Furosemide	8.0	--
Furosemide impurity A	6.65	0.83
Furosemide impurity C	4.34	0.54
Unknown impurity 1	12.01	1.5

Validation of the Method

➢ **Limit of Detection**

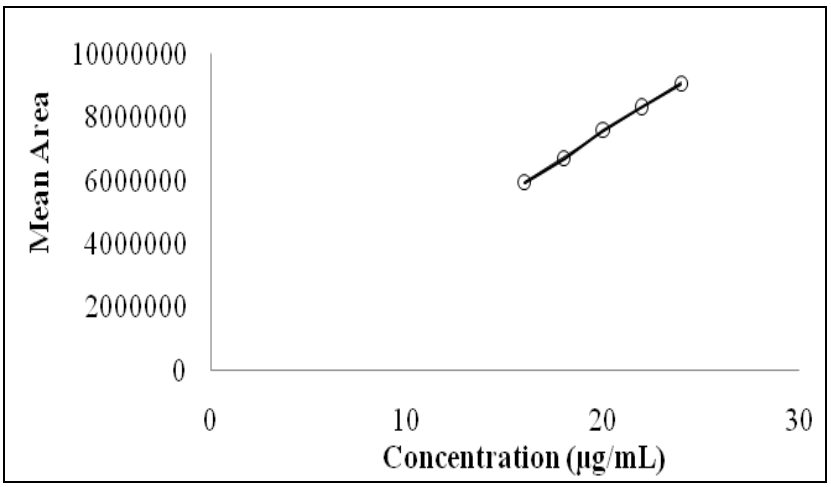
The LOD was found to be 0.40µg/mL. The result indicates an acceptable level of precision at the LOD level for the analytical system.

➢ **Limit of Quantification**

The LOQ was found to be 1.23µg/mL. The result indicates an acceptable level of precision at the LOQ level for the analytical system.

➢ **Linearity**

The polynomial regression data for calibration plots (n=3) showed a good linear relationship over concentration range of 4 to 9.3 µg/mL. Coefficient of correlation (r) was 0.999 (acceptance criteria = 0.995) with slope of 0.003 and intercept of 0.52. No significant difference was observed in the slopes of standard curve. Table 2 represents the linearity of Furosemide response.



Correlation	0.999
Slope	3924
Y intercept	-33365
Straight line equation	y = 3924x - 33365

Figure: 7. Linearity of Furosemide response by HPLC

There was a good correlation of data over the range 0.016mg/mL to 0.024mg/mL of furosemide.

➢ **Accuracy**

Table: 2. Accuracy of HPLC method for estimation of Furosemide

Theoretical Content (µg/ml)	Actual Content found (µg/ml)	Recovery %
80.00	80.68	100.85
80.00	80.55	100.69
80.00	80.56	100.71
100.00	101.93	101.93
100.00	102.33	102.33
100.00	101.11	101.11
120.00	119.50	99.59
120.00	120.64	100.54
120.00	119.75	99.79

The accuracy of the method was checked by recovery of drug from sample preparations, accurately spiked with different concentrations of the drug. The results indicated that there was no significant difference between the calculated percent recovery and actual value drug. Percent RSD was found to be less than 2.

➢ **Precision**

System Precision

Table: 3. Furosemide Standard solution (n=6)

Sr. No	Peak area
1	7666165
2	7680923
3	7682375
4	7702819
5	7686817
6	7666165
%RSD	**0.90**

Repeatability

Table: 4. Instrumental precision data of HPLC studies (n=3)

	Precision day 1	Intermediate precision day 2
Mean (%)	101.0	101.4
Minimum (%)	99.6	100.8
Maximum (%)	102.22	102.3
Standard deviation	0.9428	0.5
CV (%)	0.9338	0.4933

Table: 5. Precision data of reproducibility of HPLC studies (n=12)

	Precision day 1	Intermediate precision day 2
Mean (%)	101.0	101.4
Minimum (%)	99.6	100.8
Maximum (%)	102.22	102.3
Standard deviation	0.9428	0.5
CV (%)	0.9338	0.4933

The results along with the percent RSD, assay of drug shown indicates an acceptable level of precision for the analytical system for each day less than 2%.

- **Robustness**

The method was found to be robust in terms of small change in flow rate, change in mobile phase pH and change in composition of mobile phase as % RSD between normal conditions and altered conditions was not more than 2.0%.

Table: 6. Robustness of HPLC method for estimation of Furosemide

Altered condition	Area (Mean)	% Assay	Absolute Difference	RSD	Tailing factor	Retention time
Change in flow rate						
Unaltered Condition						
Flow rate 1.0 mL/min	7776741	100.21	--	0.07	1.19	7.855
Altered Condition						
Flow rate 1.2 mL/min (Set 1)	6517928	100.558	0.348	0.27	1.17	6.615
Flow rate 0.8 mL/min (Set 2)	9683727	100.84	0.63	0.24	1.23	9.501
Change in composition						
Unaltered Condition						
Buffer (70): 1-propanol (30)	7776741	100.21	--	0.07	1.19	7.855
Altered Condition						
Buffer (67): 1-propanol (33)	7895438	100.54	0.33	0.49	1.28	7.77
Buffer (73): 1-propanol (27)	7441076	100.42	0.21	1.12	1.19	10.97
Change in mobile phase pH						
Unaltered Condition						
pH 7.0	7776741	100.219	-	0.07	1.19	7.855
Altered Condition						
pH 6.8	8290188	100.309	0.09	0.01	1.28	7.972
pH 7.2	8346930	101.014	0.79	0.10	1.17	7.969

The system suitability parameter like the percent RSD of area and tailing factor was not significantly changed with altered conditions. The method was found to be robust in terms of small change in composition of mobile phase and flow rate of mobile phase.

- **Solution stability**

Table: 7. Solution stability of Furosemide

Time (Hour)	Test sample			
	Room temperature		Below 10 °C	
	%Assay	% Assay Difference w.r.t. to initial	%Assay	% Assay Difference w.r.t. to initial
Initial	98.61	-----	98.61	-----
12	99.89	0.28	100.04	1.43
24	99.92	0.31	100.27	1.66

The % assay difference in Test solution was found to be 1.43 % and 1.66 % at 12 hr and 24 hr respectively when stored at below 10 0C while 0.28 % and 0.31% at 12 hr and 24 hr respectively when stored at room temperature which is well within the acceptance criteria of not more than 2.0%. Based on the above data it can be concluded that the test solution stored at below 10 0C and at room temperature showed better similar results. Test solution can be used up to 24 hr after preparation when stored in refrigerator.

Assay of Furosemide Tablets

Table: 8. Peak area of Furosemide standard and sample

Injection	Sample area	Standard area	% Assay
1	7990023	7966218	100.46
2	7994479	7966218	100.51
3	8005443	7966218	100.65

The assay of the tablets was within the acceptable limit (between 90% and 110%).

CONCLUSION

Thus stress degradation studies were carried out and a simple, precise, accurate and selective method was successfully developed and validated for quantitation of Furosemide as per ICH guidelines. The method is stability-indicating and can be used to assess the short term and accelerated stability of Furosemide in the bulk drug as well as formulation. The method can be conveniently used for assay of Furosemide in the bulk drug and in pharmaceutical dosage forms in pharmaceutical industry.

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