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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OFFRUSEMIDE IN FRUSEMIDE INJECTION BY HPLC TECHNIQUE

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ABSTRACT

Frusemide injection is used to help treat fluid retention (edema) and swelling that is caused by congestive heart failure, liver disease (cirrhoiss), kidney disease, or other medical conditions. Then a new, precise and accurate high performance liquid chromatographic method has been developed for the simultaneous determination of frusemide in pharmaceutical preparations. The method was developed using an Inertsil C18 (250 x 4.6mm), 5µ column with a mobile phase consisting of Methanol:Water (70:30 v/v),pH 3.20 ±0.05, which was adjusted by o-phosphoric acid at a flow rate of 1.0 ml/min and detection was carried out at 236 nm. The developed HPLC method was validated with respect to linearity, accuracy, specificity, limit of detection, limit of quantification, precision, robustness.[1,2,4]

KEYWORD: Frusemide, Method Development, HPLC, Validation.

INTRODUCTION

Frusemide (Lasix) Injection belongs to a group of medicines called diuretics. It is used for the emergency treatment of very high blood pressure (hypertensive emergency), as it reduces the blood pressure immediately. It also rapidly reduces the swelling (edema) caused by too much water in the body. Lasix Injection helps your body get rid of extra water and salt through urine. It is commonly used to treat people with excess fluid build-up due to heart failure, liver or kidney disease. It is injected into a vein under the supervision of a doctor. Your doctor

may monitor your blood pressure after giving this medicine. Common side effects of this medicine include dizziness, weakness, dehydration, decreased potassium level in blood, increased blood uric acid, decreased magnesium level in blood, and increased thirst. These are usually mild and disappear after a short time. Consult your doctor if they bother you or do not go away.

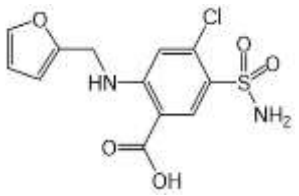


Fig. Structure of Furosemide.

Frusemide, commonly known by its brand name Lasix, is a potent diuretic medication used to treat various conditions associated with fluid retention, such as edema and hypertension. The mechanism of action of Lasix injection is essentially the same as that of furosemide, the active ingredient.

Here's a detailed explanation of how Lasix injection works:

1) Inhibition of Sodium-Potassium-Chloride Symporter (NKCC2): Lasix primarily acts on a specific part of the kidney called the thick ascending limb of the loop of Henle. Within this segment, it inhibits the sodium-potassium-chloride cotransporter (NKCC2) located on the luminal membrane of cells. By blocking NKCC2, Lasix interferes with the normal reabsorption of sodium, potassium, and chloride ions from the urine back into the bloodstream. This disruption of ion transport prevents the establishment of an osmotic gradient necessary for water reabsorption, thereby promoting diuresis.
2) Increased Excretion of Sodium, Chloride, and Water: As a consequence of inhibiting NKCC2, Lasix promotes the excretion of sodium, chloride, and potassium ions into the urine. This increase in ion excretion is accompanied by the osmotically driven excretion of water. Consequently, urine volume increases, leading to the removal of excess fluid from the body, which is beneficial in conditions like congestive heart failure, liver cirrhosis, and renal impairment.
3) Vasodilation: Lasix also possesses mild vasodilatory properties, particularly on the veins. By dilating blood vessels, Lasix reduces venous pressure and preload on the heart. This effect can be helpful in conditions where reducing blood volume and alleviating pressure on the cardiovascular system are therapeutic goals.

4) Rapid Onset and Short Duration: When administered intravenously as an injection, Lasix exhibits a rapid onset of action, typically within minutes. Its effects on diuresis and fluid balance are relatively short-lived, making it suitable for acute management of conditions requiring prompt diuresis.

In summary, Lasix injection exerts its therapeutic effects primarily by inhibiting the reabsorption of sodium, chloride, and potassium ions in the loop of Henle, leading to increased urine production and reduction of fluid volume in the body. Its vasodilatory properties further contribute to its clinical efficacy in managing conditions associated with fluid overload and hypertension.

Duration of action

The duration of action of intravenous (IV) furosemide (Lasix) can vary depending on factors such as the individual's renal function, dosage, and underlying medical conditions. Generally, the onset of action for IV furosemide is quite rapid, typically within minutes of administration, with peak effects occurring within 30 minutes to an hour. The duration of action is usually around 2 to 3 hours, but this can vary among patients.

MATERIAL AND METHODS

Method Development

Analytical method development and validation play important roles in the discovery development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of drug product There are many factors to consider when developing methods. The initially collect the information about the analyte’s physiochemical properties (piker, log P,solubility) and determining which mode of detection would be suitable for analysis in case of UV detection). The majority of the analytical development effort goes in validating a HPLC–method. There are many steps involve in method development which are:

- Physicochemical properties of drug
- Set up HPLC conditions
- Sample preparation
- Method optimization
- Validation of developed method

METHOD VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used foranalyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH.

VALIDATION PARAMETER

The following are typical analytical performance characteristics which may be tested duringmethods validation

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Robustness
- System suitability determination

FORMULATION DETAILS OF FRUSEMIDE(LASIX INJECTION)

Manufactured by : Sanofi India ltd.

Batch no : 2123231

Mfg. Date : DEC. 2023

Exp Date : NOV. 2026

Process of analysis

Phosphate Buffer (pH 4.0): 4.08 gm of Potassium Di-hydrogen Orthophosphate dissolved anddiluted in 1000 ml of purified water. Adjust the pH to 4.0 with diluted Orthophosphoric acid.

Mobile Phase- Phosphate Buffer (pH 4.0) (40): Acetonitrile (40): Methanol (20). Mixed, Sonicated and filtered through 0.45 micron nylon filter paper.

Chromatographic Condition

Column: 250 x 4.6mm, 5 µm, Agilent Eclips C18

Wavelength: 254 nm

Flow Rate: 1.0 ml / min.

Injection Volume: 20 µl

Column Oven Temperature: 40 ^{0}C

Run time: 7 min

Mobile Phase: Phosphate Buffer: Acetonitrile: Methanol (40:40:20)

STANDARD PREPARATION

Weigh accurately and transfer about 40 mg Frusemide Standard in 100ml volumetric flask. Add about 70ml of mobile phase. Sonicate for 5 min. Allow the solution to attend room temperature and dilute upto mark with mobile phase.[7]

SAMPLE PREPARATION

Withdrawn all content of solution via suitable Hypodermic needle and syringe by cutting ampoule on appropriate position and transfer into 100 ml volumetric flask, Dilute with mobilephase. And inject.

Inject Blank, standard and Sample preparation as sequence given below, (1) Blank (2) Standard preparation (5 replicates) (3) Blank (4) Sample preparation (5) Standard preparation (Bracketing standard) (7)

SYSTEM SUITABILITY CRITERIA

1) % RSD for retention time of replicates of standard preparation should not be more than 1.0 %.
2) % RSD for area of replicates of standard preparation should not be more than 2.0%
3) Theoretical plates for all standards injections should not be less than 2000. Report

Theoreticalplates of first replicate of standard preparation.

4) Tailing factors for all standard injections should not be less than 2.0. Report tailing factor offirst replicate of standard preparation.
5) Cumulative % RSD for retention time of replicates of standard preparation with retentiontime of bracketing standard should not be more than 1.0 %.
6) Cumulative % RSD for area of replicates of standard preparation with area of bracketing standard should not be more than 2.0 %. If system suitability found within limit, perform calculations for content of Frusemide Injection by using formula,

Calculate Content of Frusemide Injection (mg / ml) for sample preparations independently by using formula described below.

$$= \frac{\text{(Area of Frusemide Injection in sample preparation)}}{\text{(Area of Frusemide in Standard preparation)}} \times \frac{W1(mg)}{100} \times \frac{100}{1} \times \frac{P}{100} \times$$

Where,

W1 = Weight of Frusemide standard taken for preparation of standard preparation (in mg)P = % Purity Frusemide Standard

Acceptance Criteria: Each Injection: Frusemide per Injection = 36 mg to44 mg

% Assay = 90.00 % to 110.00%

DEVELOPMENT OF HPLC ANALYTICAL METHOD

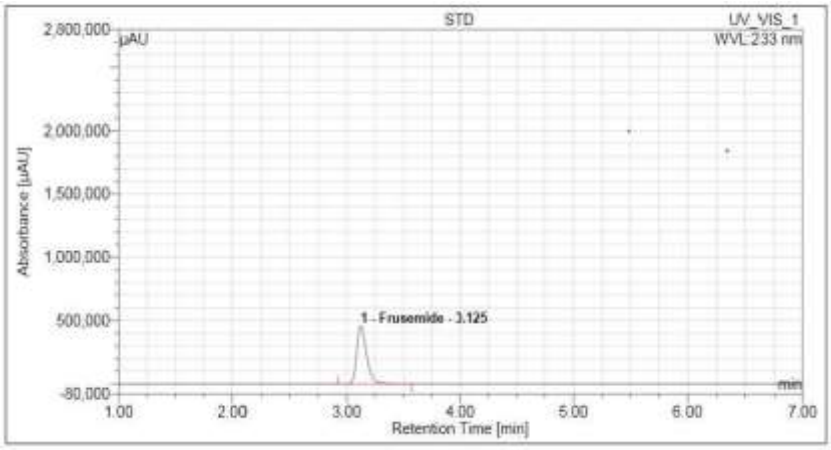
HPLC Used : Low Pressure Quaternary Gradient

(Make-Shimadzu, Model-LC2010)

HPLC column- 250 x 4.6mm, 5um, Agilent Eclips C18.

Sr no.	Chemical used	Make	Grade
1	Potassium Di- hydrogen Orthophosphate	Fisher scientific	SQ grade
2	Acetonitrile	Merck	Hplc grade
3	Orthophospric acid	Rankem	AR grade
4	Purified water	--	Mili Q

METHOD DEVELOPMENT DETAILS

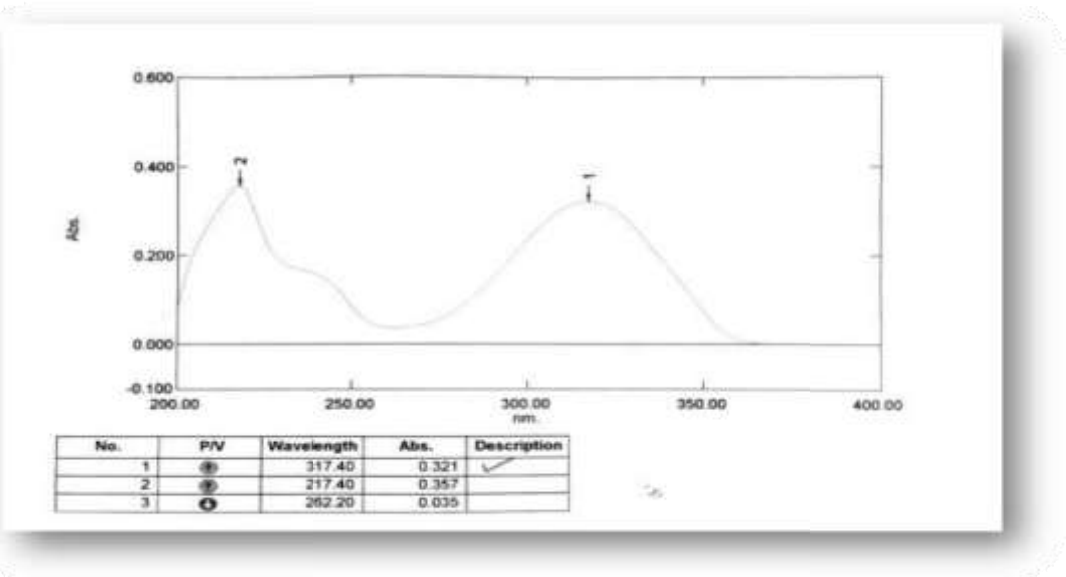


TRAIL 1

Initially a mobile phase consisting of 10 mM Potassium Di-hydrogen Orthophosphate (pH 3.5): Methanol (60:40 %v/v) at a flow rate of 1.0 mL/min was used on an Agilent Eclipse C18 column (250 x 4.6) 5micron column at ambient temperature using mobile phaseas diluents. Frusemide did not elute under these conditions.

Following changes are done

1) Methanol solvent is introducted in some portion of acetonitrile.
2) Buffer: ACN: methanol (40:40:20).
3) Conc. of salt increased from 20 to 30mM
4) pH of buffer is shifted from 3.5 to 4.0
5) Column length is decreased. From 250 to 150 cm, Lambda max is confirmed by ultravioletspectroscopy. Spectra is observed as below.[11]

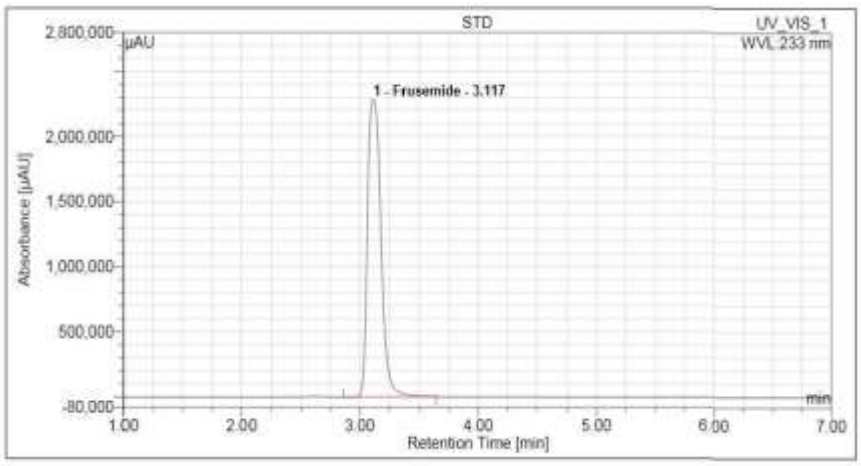


METHOD VALIDATION

A) SPECIFICITY AND SYSTEM SUITABILITY

Specificity demonstrated by observing interference of mobile phase (Diluent).

System suitability parameters (%RSD of area, Retention time, Theoretical Plates andTailing factor) demonstrated by injecting standard preparation in replicate.1.

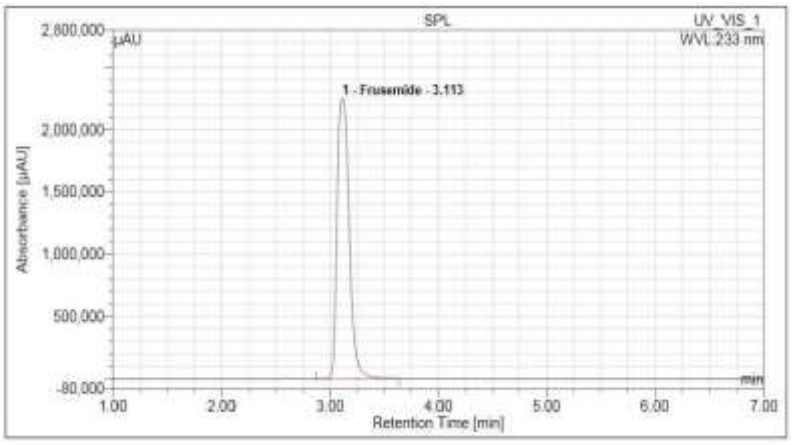


Std InjNo.	Retentiontime	Area	Theoreticalplates	Tailingfactor
1	3.12	302451.584	3528	1.34
2	3.12	302044.337	3547	1.36
3	3.12	301679.120	3560	1.35
Average	0.0312%	3020.583%	35.45%	

A) PRESCISION

a) REPEATABILITY

The repeatability was demonstrated by preparing the standard solution at 40 ppm concentration and six independent consecutive sample preparation at 40 ppm. System suitability found within limit. Relative standard deviation of assay value for five preparations found within 2 %.



Std InjNo.	Retentiontime	Area	Theoreticalplates	Tailingfactor
1	3.11	294935.854	3627	1.35
2	3.11	293997.981	3638	1.35
3	3.11	294399.948	3627	1.35
4	3.11	294259.602	3638	1.36
5	3.11	293643.222	3657	1.35

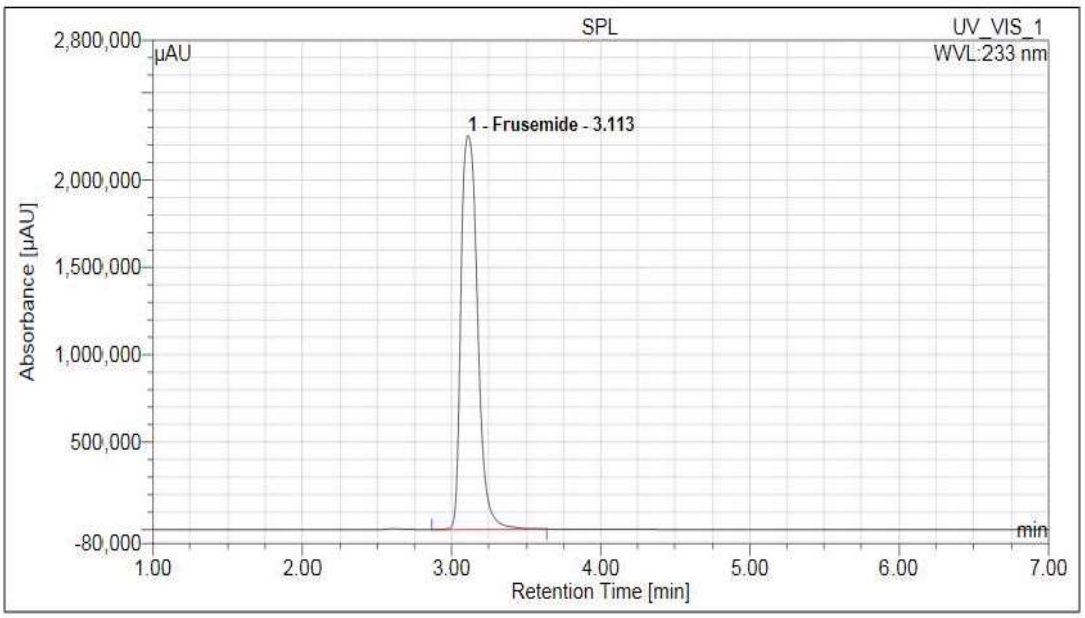
Sample	RT	Area	% Assay
1	3.11	294935.854	100.84
2	3.11	293997.981	100.52
3	3.11	294399.948	100.65
4	3.11	294259.602	100.61
5	3.11	293643.222	100.39

% RSD=0.18

Conclusion of repeatability- % RSD for repeatability found 0.18 which well within limit therefore method is repeatable**.**

B) INTERMEDIATE PRECISION: The Intermediate Precision was demonstrated by preparing the standard solution at 40 ppm concentration and six independent consecutive sample preparation at 40 ppm. by other person on other day with other set of chemicals. System suitability found within limit. Relative standard deviation of assay value for six preparations found within 2%.

% Variation of average assay values obtained via repeatability and intermediate precision found within 3 %.



Std InjNo.	Retentiontime	Area	Theoreticalplates	Tailingfactor
1	3.11	295624.468	3607	1.36
2	3.11	294305.274	3637	1.35
3	3.11	294203.750	3628	1.36
4	3.11	294153.820	3628	1.35
5	3.11	294845.209	3627	1.36
6	3.11	295512.898	3601	1.35

Sample	RT	Area	% Assay
1	3.11	295624.468	100.58
2	3.11	294305.274	100.13
3	3.11	294203.750	100.10
4	3.11	294153.820	100.08
5	3.11	294845.209	100.32
6	3.11	295512.898	100.55

Conclusion of Intermediate precision

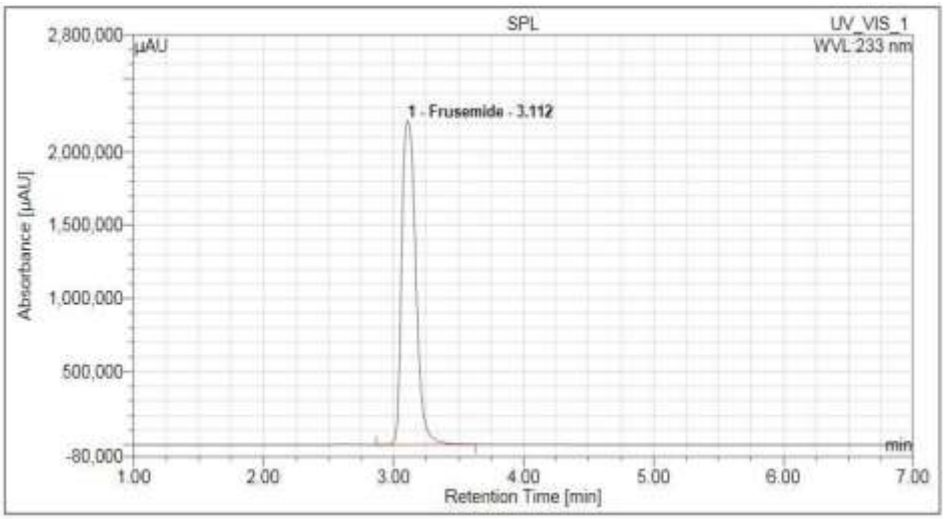
% RSD for 6 replicate independent analysis by changing various objects found 0.23% which well within limit therefore method is intermediately reputable.

% RSD for 12 observations (6 of repeatability and 6 of intermediate precision) found 0.24% which well within limit therefore Based on both experiment Method found Precise.

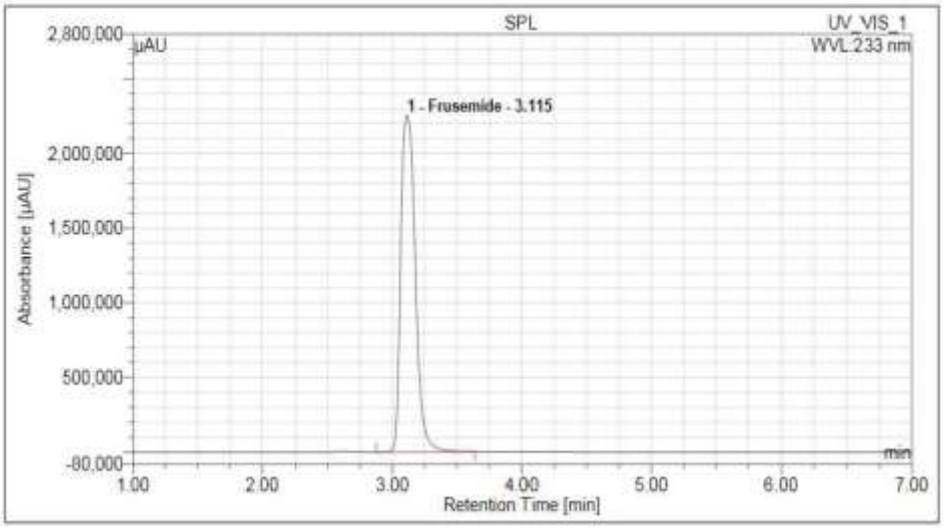
c) ACCURACY

To determine the accuracy of the method, recovery studies were carried out in triplicate by using different concentrations of pure drug in the preanalyzed samples with 3 different concentrations of sample that consists of 80%, 100% and 120% of the pure drug. The accuracy was expressed as the percentage analytes recovered.

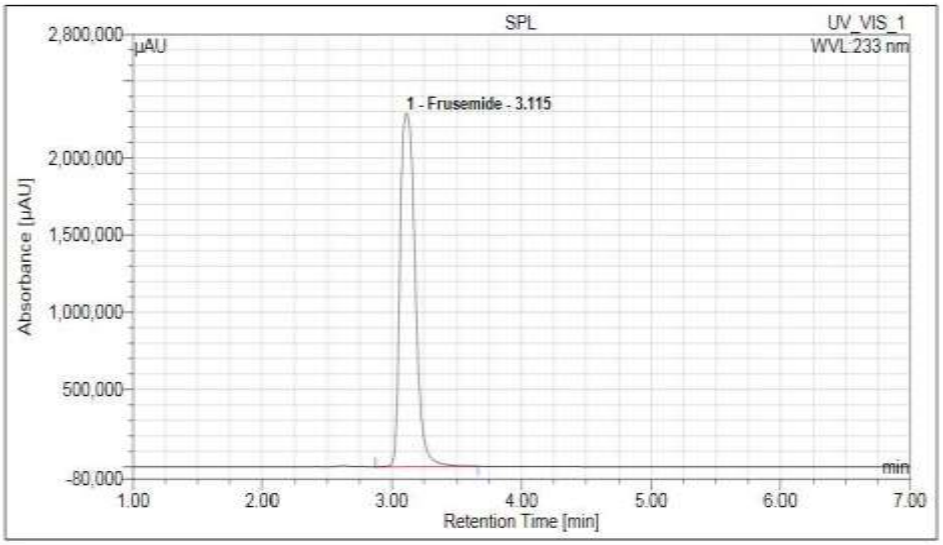
ACCURACY SAMPLE 80%



ACCURACY SAMPLE 100%



ACCURACY SAMPLE 120%



Sample	Area Frusemide	% Assay Frusemide
Spl-80%	287117.9012	98.86
Spl-80%	288123.9558	99.20
Spl-80%	282012.9546	97.10
Spl-100%	302714.6572	104.23
Spl-100%	302458.2546	104.14
Spl-100%	302586.5264	104.18
Spl-120%	317498.0421	109.32
Spl-120%	314565.2546	108.31
Spl-120%	312545.2351	107.61

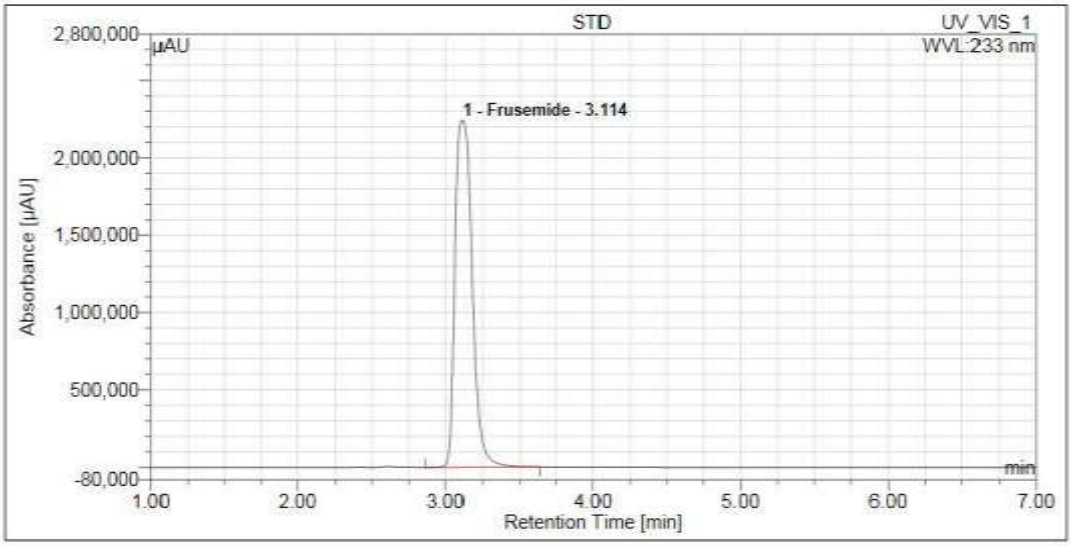
D) LINEARITY AND RANGE

From the standard stock solution, the various dilutions of Frusemide in the concentration of

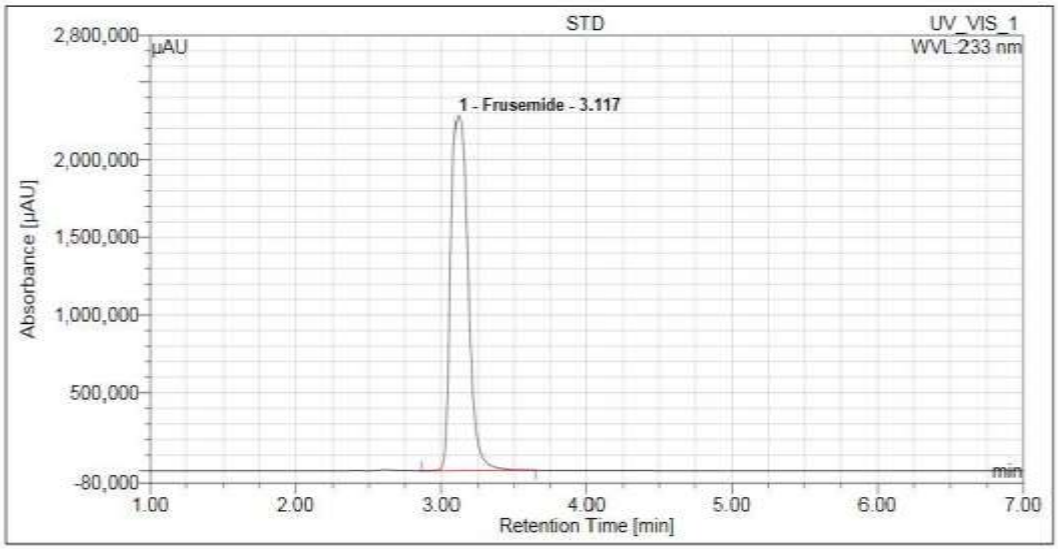
163.0, 201.0, 240.0 ppm three level standard solutions of each were prepared. The solutions were injected using 20 µL injection volumes into the chromatographic system at the flow rate of 1.0 ml/min and the effluents were monitored at 233 nm, chromatograms were recorded.

Calibration curve of frusemide was obtained by plotting the peak area ratio versus the applied concentrations of frusemide by using average of each sample. The linear correlation coefficient (R2) was found to be **1.000** & %y intercept is **-0.0034%.**

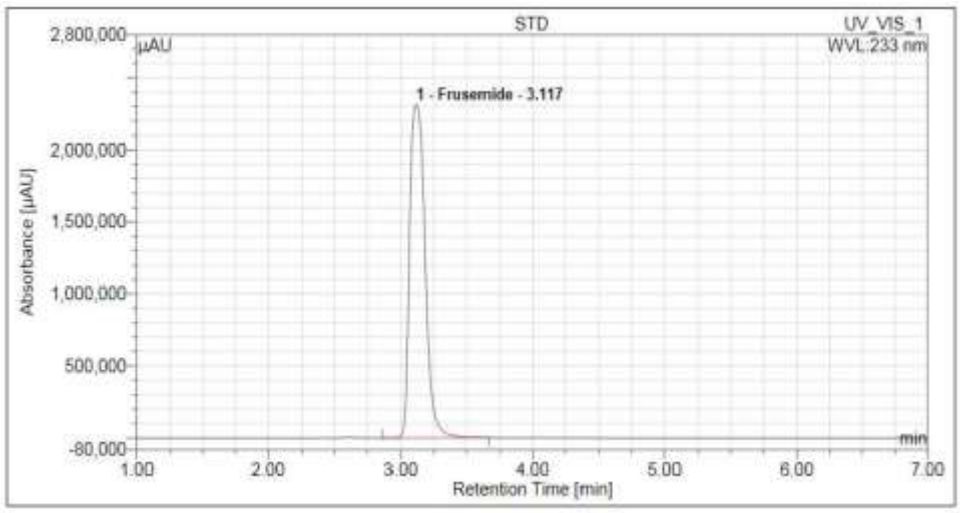
LINEARITY STD 80%



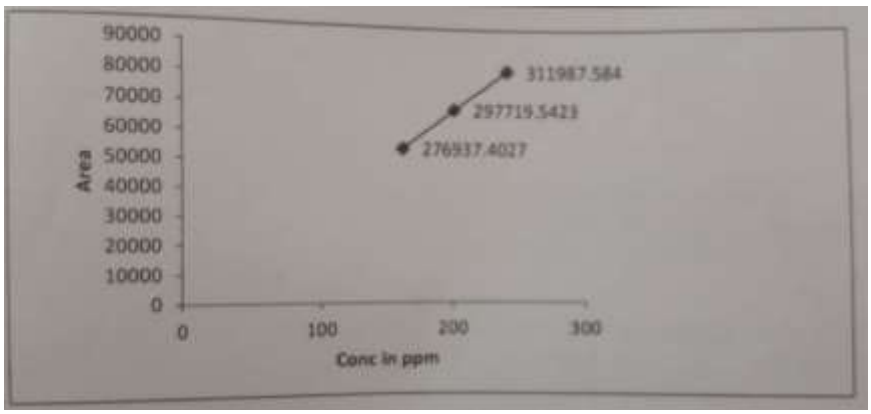
LINEARITY STD 100%



LINEARITY STD 120%



Sr No.	Conc.ppm	Area	Average
1	163.0	279541.503	276937.4027
2	163.0	275425.121	
3	163.0	275845.584	
4	201.00	2958848.110	297719.5423
5	201.00	299031.054	
6	201.00	298279.463	
7	240.00	310749.741	311987.584
8	240.00	314998.490	
9	240.00	310214.521	



Correlation -1.000

% Y intercept = 0.0034

Conclusion – Method found Linear within the range 80 % to 120 % of working level.

E) LIMIT OF DETECTION AND LIMIT OF QUANTITATION(LOD & LOQ)

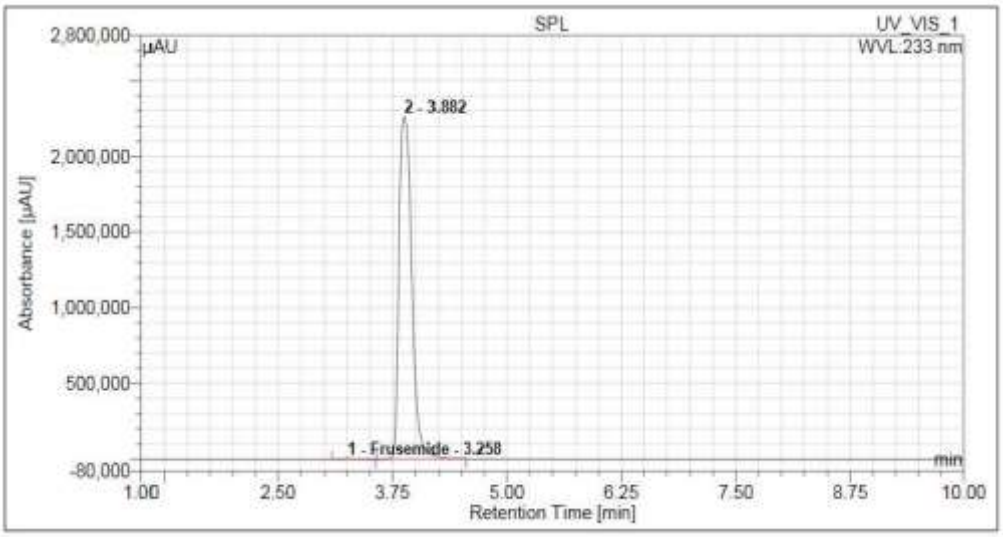
The limit of detection and limit of quantification means the lowest concentration of analytes inthe sample are detected and quantified. LOD and LOQ was found as listed below

Table of Limit of Detection & Limit of Quantitation.

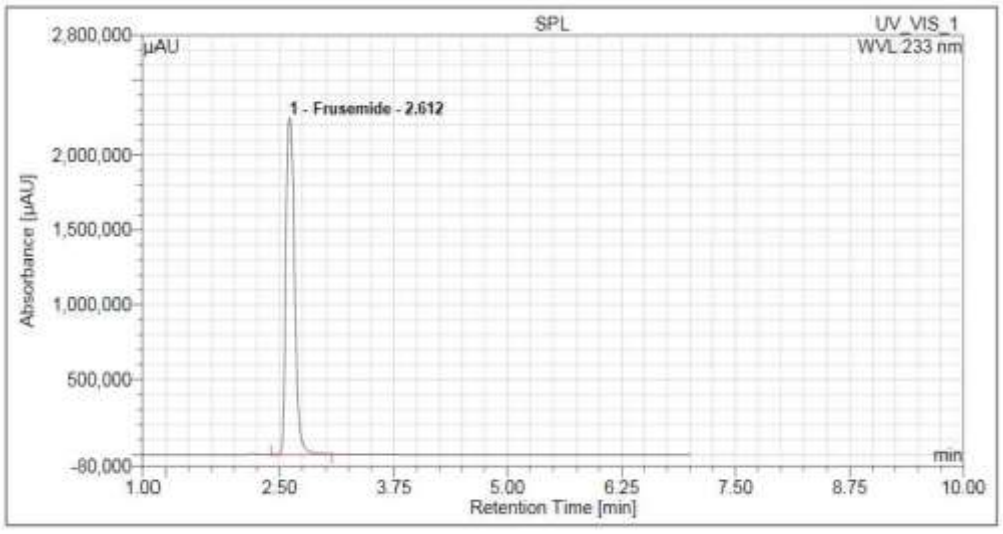
Parameter	Obtained value
LOD	4.5 ppm
LOQ	13 ppm

F) ROBUSTNESS

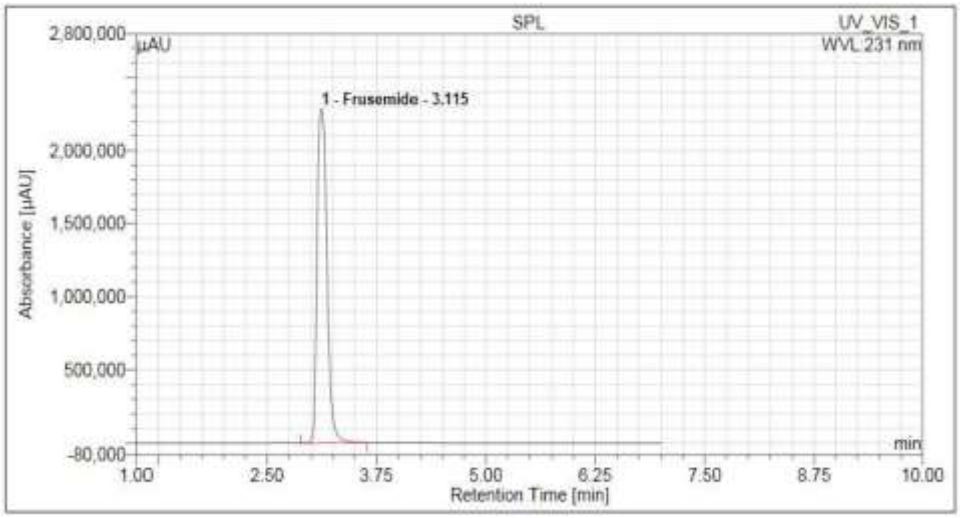
Robustness of the method was determined by intentionally changing some operating conditions such as flow rate and wavelength. The flow rate as per the developed method is 1.0 ml / min. It has been purposely changed to 0.8 ml/min and 1.2 ml/ min and the chromatogram was developed as well as the wavelength of developed method is 233 nm. It has been purposelychanged to 231 nm and 235 nm and the chromatogram was developed.



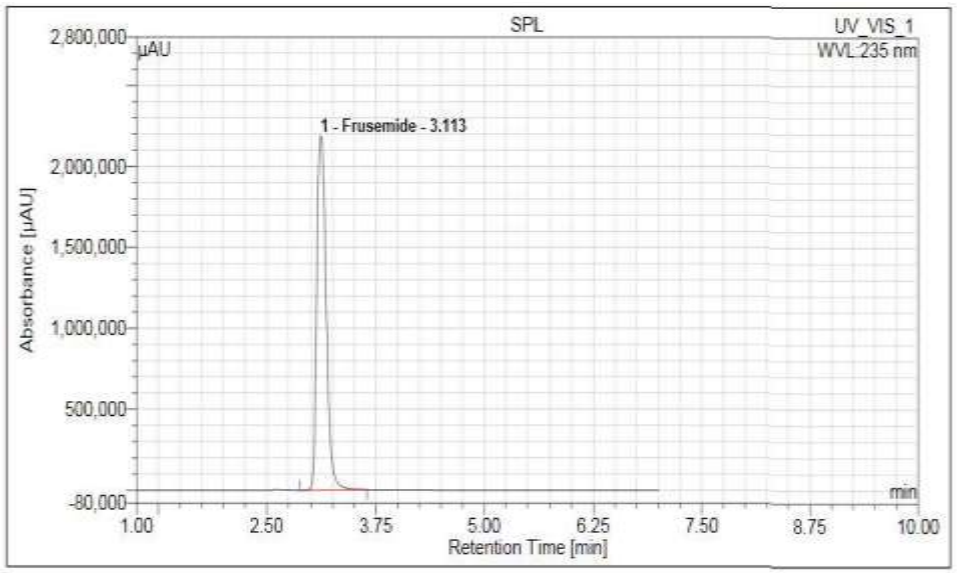
SPL Low Flow Rate = 0.8ml



SPL High Flow Rate = 1.2ml



SPL Low Wavelength = 231nm



SPL High Wavelength = 235nm

Parameter	% Assay	Cumulative % RSD withrepeatability
	Frusemide	Frusemide
Change in flow rate 0.8ml	103.02	1.14
Change in flow rate 1.2ml	99.50	0.70
Change in wavelength256nm	101.74	0.59
Change in wavelength269nm	100.93	0.23

Conclusion – Method found Robust.

RESULT AND DISCUSSION

This developed and validated HPLC assay method of frusemide is reliable and economical. Using Phosphate buffer (pH 4.0): Acetonitrile: Methanol (40:40:20) as a Mobile phase and

1.0mL/min flow rate at room temperature, chromatographic separationwas obtained on a (4.6 x 250mm 5 um Agilent Eclips C18) C18 column. The injection Volume is 20 microliter, column temperature 40^0 C and the run time is 7 minutes. The wavelength of detection is set to 233nm. The linear correlation coefficient (R2) was found to be 1.000, with a 0.0034 percent y intercept. The detection limit was determined to be 4.5 ppm while the quantification limit was found to be 13 ppm. Frusemide was proven to be 99.85% pure. The requirements for repeatability and precision have been met. For the determination of Frusemide in pharmaceutical dosage forms, the approach is simple, specific, precise, durable, and accurate(tablets & injections).

CONCLUSION

This analytical method used for determination of assay of Frusemide (lasix injection) from drug formulations like injection. This HPLC method shows all results within acceptance criteria for the analytical parameters such as Specificity and system suitability, Linearity and Range, Precision, Accuracy and Robustness. Hence method stands validated and can be used for assay analysis of frusemide from drug formulations. I suggest you can also try this method for bio-analysis of frusemide.

ACKNOWLEDGEMENT

At the very outset, I fail to find adequate words, with limited vocabulary at my command, to express my emotions to “Dear God”, & Chatrapati Shivaji Maharaj whose eternal blessings, divine presence, and masterly guidance helps me to fulfill all my goals.

It’s not easy to express my emotions in words especially when I have to say thanks to my guide Dr. Avinash M. Bhagwat (M. Pharm, PhD & HOD of YSPM’s YTC, Satara), Dr. Ajit Akal, and Sonali T. Dhumal (Managing Director and Assistance, Insta Vision Laboratory, Satara) for his inspiring guidance, affectionate encouragement, and never-ending enthusiasm; without which this research work would not have seen the light of the day.

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