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RESEARCH ARTICLE
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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-UPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF TORSEMIDE AND SPIRANOLACTONE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

Lalitha Repudi[1*], M.Lakshmi Surekha[2]

[1*]Research Scholar, School of Pharmacy, Career Point University, Kota, Rajasthan, India.
[2] Professor & Dean, Head, Department of Pharmaceutical Analysis, A.M.Reddy Memorial College of Pharmacy, Vinukonda Road, Petlurivaripalem, Narasaraopeta, Andhra Pradesh 522001, India.

***Corresponding author:-** Lalitha Repudi
*Research Scholar, School of Pharmacy, Career Point University, Kota, India.

Abstract
Simple precise and accurate method was developed for the estimation of Torsemide and Spiranolactone. The mobile phase consisting 20% Buffer: 80%ACN. The column was used: Inertsil ODS 4.6*210mm, 5µ with flow rate 1ml/min using PDA detection at 235 nm. The estimation of Torsemide and Spiranolactone was done by RP-HPLC. The assay of Torsemide and Spiranolactone was performed with tablets and the % assay was found to be 99.65 and 100.07 which shows that the method is useful for routine analysis. The linearity of Torsemide and Spiranolactone was found to be linear with a correlation coefficient of 0.998 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.1 and 0.7 for Torsemide and Spiranolactone which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.2 and 0.2 for Torsemide and Spiranolactone which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 98.0% - 101.0%. The total recovery was found to be 99.69% and 99.39% for Torsemide and Spiranolactone. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ are 3 and 10. The LOD and LOQ for Torsemide were found to be 2.98 and 10.00 and LOD and LOQ for Spironolactone were found to be 3.00 and 9.98.The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

Keywords: RP-UPLC ; Torsemide, Spiranolactone ; PDA Detection; forced degradation studies.

Introduction

Torsemide(Fig.01) is aniline pyridine sulfonyl urea derivative which is used as high ceiling diuretic and cardiovascular agent by inhibiting Na+/K+ ions. Spironolactone (fig 02) is a steroidal derivative which act as potassium-sparing diuretic. Diuretics are the agents which will increase the rate of urination and sodium excretion and are used to adjust the volume and / or composition of

body fluids in variety of clinical situations including hypertension, heart failure, renal failure, nephrotic syndrome and cirrhosis.

Literature survey revealed that several analytical methods were developed for the determination of different class of diuretics. But there is no official compendial method for the simultaneous determination of Torsemide and Spironolactone. The present study deals with simultaneous estimation of Torsemide and spironolactone by RP-UPLC. All the validation parameters related to this study were evaluated according to ICH guidelines Q2B.

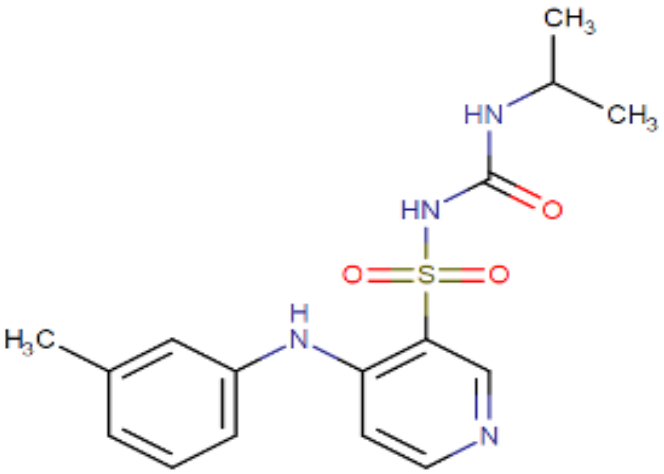

Fig.01. Chemical structure of Torsemide

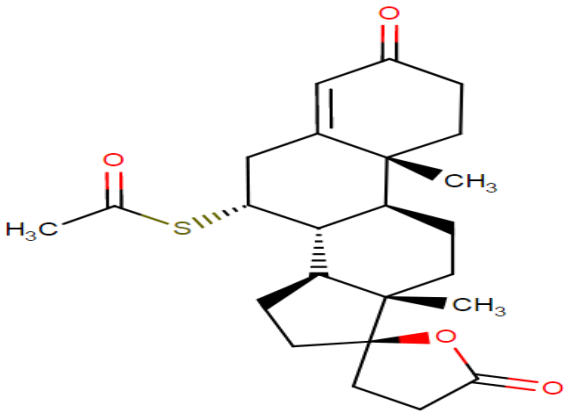

Fig.02. Chemical structure of Spironolactone

MATERILAS AND METHODS

Drugs and Chemicals

Acetonitrile, methanol, and water were purchased from Merck (Mumbai, India). All other reagents used were of HPLC grade. Standard bulk drug samples of Torsemide and Spironolactone were provided by Lupin labs Ltd., India. DYTOR PLUS 5 tablets (CIPLA LTD., TOR 5mg + SPI 50 mg) were purchased from Appollo pharmacy.

Instrumentation And Chromatographic Conditions:

Agilent 1220 LC UPLC system, EZChrome Elite software with UV/VIS detector. Separation was carried out by using mobile phase consisting 20:80 (v/v) 10mM potassium dihydrogen Ortho phosphate and acetonitrile with 1.0 ml/min flow rate. All weights were taken using SHIMADZU balance of model ATX224.

Table 1: Instruments used

SL. No	Instrument	Model
1	UPLC	WATERS, Acquity Model with PDA Detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000^{+}
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

Table: 02 . Chemicals used

SL.No	Chemical	Brand
1	Torsemide	Supplied by Lupin
2	Spiranolactone	Supplied by Lupin
3	Ortho phosphoric acid	FINAR chemical LTD
4	Water and Methanol for HPLC	Standard solutions Ltd
5	Acetonitrile for HPLC	Standard solutions Ltd
6	HCl, H_2O_2, NaOH	MERCK

METHOD DEVELOPMENT:

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile : methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to 0.1% OPA (pH 3.0), Methanol in proportion 10: 10 v/v respectively.

Wave length selection:

UV spectrum of 10µg/ml Torsemide and 10µg/ml Spiranolactone in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 235 nm (Fig 03). At this wavelength both the drugs show good absorbance.

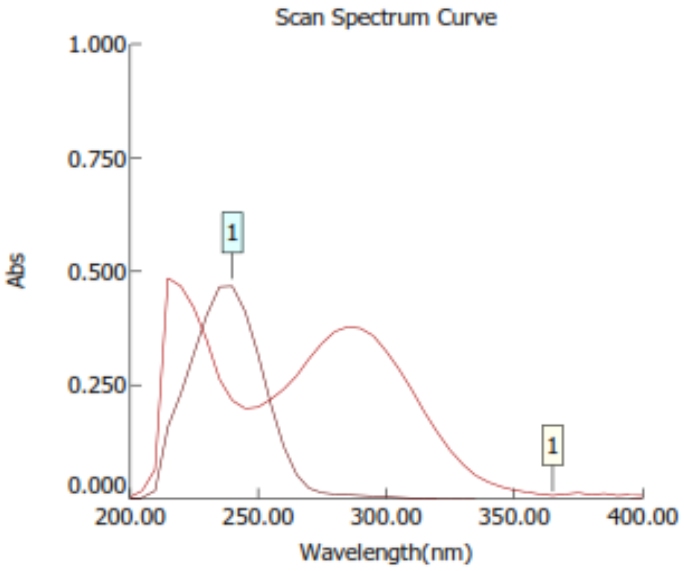

Fig.03. Ovelain spectra of Torsemide and Spironolactone

Optimization of Column:

The method was performed with various columns like C_{18} column Phenomenex column, YMC, and Inertsil ODS column. WATERS BEH (2.1 x 50mm, 3µm) was found to be ideal as it gave good peak shape and resolution at 0.25 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used: Waters UPLC with auto sampler and PDA detector.

Temperature	:	Ambient (25° C)
Mode of separation	:	Isocratic mode
Column	:	WATERS BEH (2.1 x 50mm, 3.0µm)
Buffer	:	0.1% OPA
pH	:	3.0
Mobile phase	:	Buffer: ACN (20:80)
Flow rate	:	0.25 ml per min
Wavelength	:	235 nm
Injection volume	:	4µl
Run time	:	10 min.

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown below.(Fig.4,5 & 6).

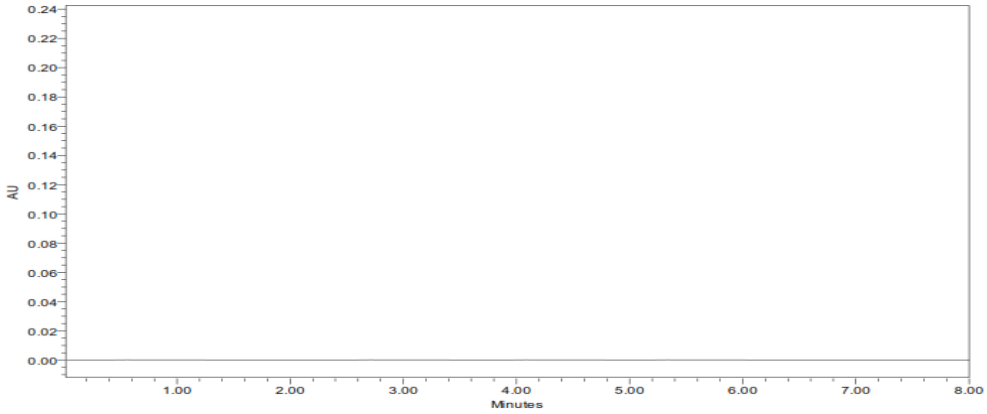

Fig.04. Chromatogram for Blank

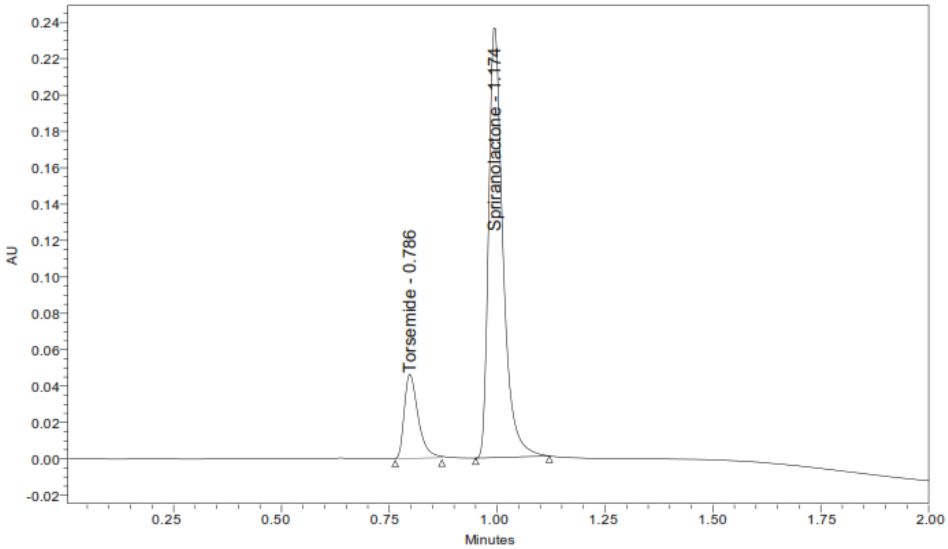

Fig.05: Chromatogram for Standards of Torsemide and Spironolactone

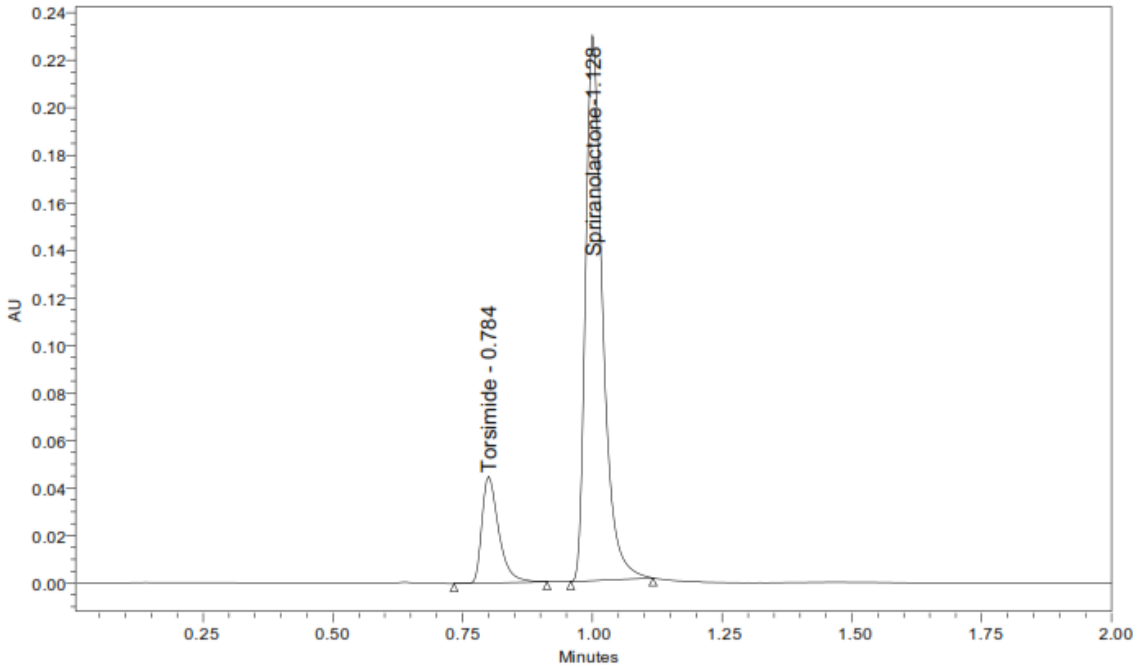

Fig.06.: Chromatogram for Sample (Torsemide and Spironolactone)

Preparation of buffer and mobile phase:

Preparation of 0.1% OPA buffer:

1 ml of OPA is taken in 1000 ml of HPLC water pH was adjusted with 0.1M NAOH up to 3.0.final solution was filtered through 0.45 µm Membrane filter and sonicate it for 10 mins.

Preparation of mobile phase: Accurately measured 200 ml (20%) of above buffer and 800 ml of Acetonitrile HPLC (10%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Preparation of Torsemide Stock Solution: Standard stock solution of Torsemide was prepared separately by dissolving 10 mg of the drug in 10 mL of methanol to get a concentration of 1000µg/ mL. One milliliter of this solution was diluted to 10mL to get a concentration of 100µg/ mL.

Preparation of Spironlactone standard stock solution:

Standard stock solution of Spironlactone was prepared by dissolving 10mg of Spironolactone in 10mL of methanol to get a concentration of 1000µg /mL. One milliliter of this solution was further diluted to 10 mL with mobile phase to get a concentration of 100 µg/ mL.

Procedure for Sample Preparation of Formulation

Details of marketed Formulation:

Brand Name	Manufactured By	Concentration of Torsemide (mg)	Concentration of Spironolactone (mg)
PLUS 5	CIPLA LTD.	5	50

Twenty tablets were weighed and powdered. A quantity of tablet powder equivalent to 10 mg of SPL was weighed and transferred to 10 mL volumetric flask containing 8 mL of mobile phase and ultra-sonicated for 20 min, and the volume was made up to the mark with mobile phase. The solution was filtered through 0.41 µ filter paper. One milliliter of this solution was further diluted to 10 mL with mobile phase to get 10 + 100 µg mL-1 concentrated TOR & SPI solution. The chromatographic conditions were set before injecting the samples into the system. After stabilizing the system, the tablet sample solution was injected into the system, a chromatogram was obtained peak areas and retention time was recorded. The process was repeated for six injections and the amount of sample was estimated from the respective calibration curves.

Validation Parameters:

ASSAY:

Standard Solution Preparation:

Accurately weigh and transfer 10 mg of Torsemide and 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 10 mg of Torsemide and 25 mg of Spiranolactone sample into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 10 mg of Torsemide and 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (50ppm & 125ppm of Torsemide &Spiranolactone):

0.5ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – II (100ppm & 250ppm of Torsemide &Spiranolactone):

1ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – III (150ppm & 375ppm of Torsemide &Spiranolactone):

1.5ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – IV (200ppm & 500ppm of Torsemide &Spiranolactone):

2ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – V (250ppm & 625ppm of Torsemide &Spiranolactone):

2.5ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Precision:

Preparation of stock Solution:

Accurately weigh and transfer 10 mg of Torsemide and 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

Intermediate Precision/Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory.

Preparation of stock solution:

Accurately weigh and transfer 10 mg of Torsemide and 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

Accuracy:
For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

Preparation of Standard stock solution:
Accurately weigh and transfer 10 mg of Torsemide and 25mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

Preparation Sample solutions:
For preparation of 50% solution (With respect to target Assay concentration):
Accurately weigh and transfer 5 mg of Torsemide and 12.50 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

For preparation of 100% solution (With respect to target Assay concentration):
Accurately weigh and transfer 10 mg of Torsemide and 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% solution (With respect to target Assay concentration):
Accurately weigh and transfer 30 mg of Torsemide and 37.5 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents

Limit of Detection:
Preparation of Torsemide solution:
Preparation of 150µg/ml solution:
Accurately weigh and transfer10 mg of Torsemide working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 4.2 µg/ml solution:
Further pipette 0.28 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Spiranolactone solution:
Preparation of 375 µg/ml solution:
Accurately weigh and transfer 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 2.66 µg/ml solution:
Further pipette 0.071 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents

Limit of Quantification:
Preparation of Torsemide solution:
Preparation of 150 µg/ml solution:
Accurately weigh and transfer 10 mg of Torsemide working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 14.04 µg/ml solution:
Further pipette 0.936ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Spiranolactone solution:
Preparation of 375 µg/ml solution:
Accurately weigh and transfer 25 mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 8.74 µg/ml solution:
Further pipette 0.233ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Robustness:
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.22 ml/min to 0.27ml/min.
Standard solution 150 ppm of Torsemide& 375 ppm of Spiranolactone was prepared and analysed using the varied flow rates along with method flow rate.
On evaluation of the above results, it can be concluded that the variation in flowrateaffected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The Organic composition in the Mobile phase was varied from ±10%.
Standard solution 150 ppm of Torsemide & 375 ppm of Spiranolactonewas prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.On evaluation of the above results, it can be concluded that the variation in 10%.Organic composition in the mobile phase affected the method significantly. Hence it Indicates that the method is robust even by change in the Mobile phase ±10

Degradation Studies:
The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Torsemide and Spiranolactone using the proposed method.fig 7,8,9 and Table 3 & 4).

Preparation of stock:
Accurately weigh and transfer 10 mg of Torsemide and 25mg of Spiranolactone working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Hydrolytic degradation under acidic condition
Pipette 1.5 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition
Pipette 1.5 ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Thermal induced degradation
Torsemide and Spiranolactoneesample was taken in petridish and kept in Hot air oven at 110^0 C fo 3 hours. Then the sample was taken and diluted with diluents and injected into UPLC and analysed.

Oxidative degradation
Pipette 1.5 ml above stock solution into a 10ml volumetric flask and 1ml of 30% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation: Pipette 0.3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

System Suitability
The system suitability was confirmed by six replicate injections of the mixture containing 16 + 160 µg mL-1 of TOR and SPI drugs. The resolution, HETP, number of theoretical plates, peak asymmetry, were calculated from obtained chromatogram. The standard deviation was found to be 0.42 for TOR and 0.84 for SPI respectively. The representation chromatogram for standard solution of mixture is shown in *Fig.1*.

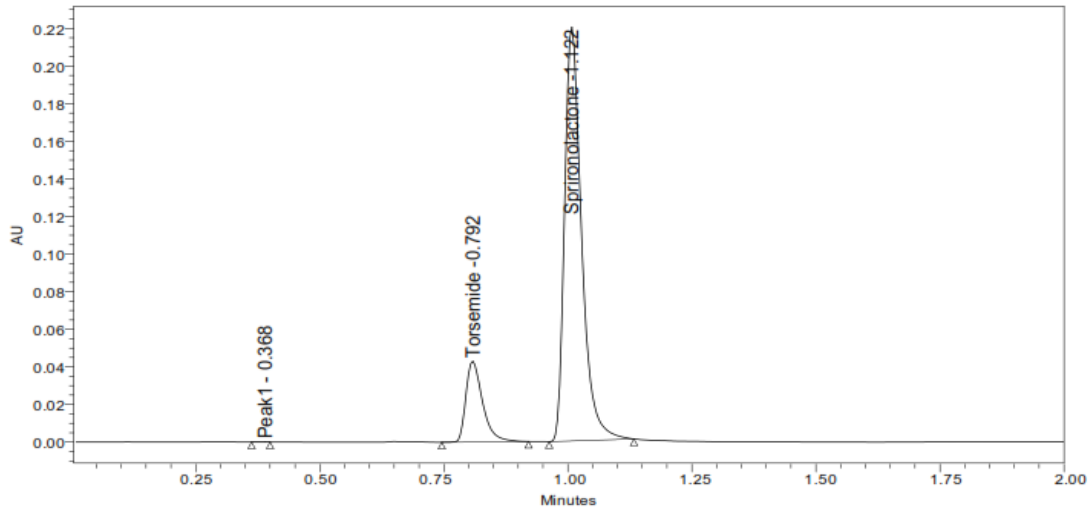

Fig.7: Chromatogram showing Acid

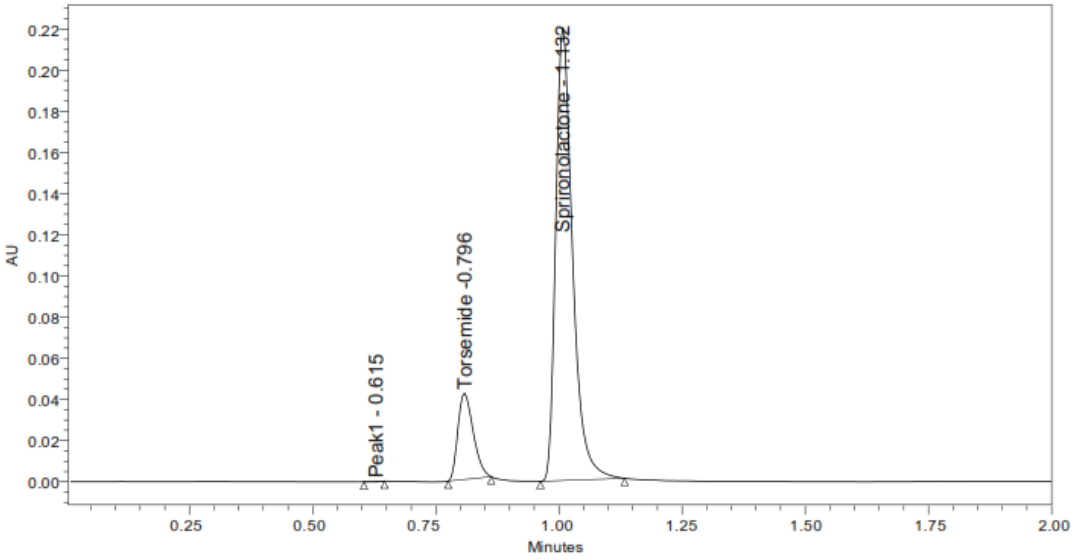

Fig.8: Chromatogram showing Base

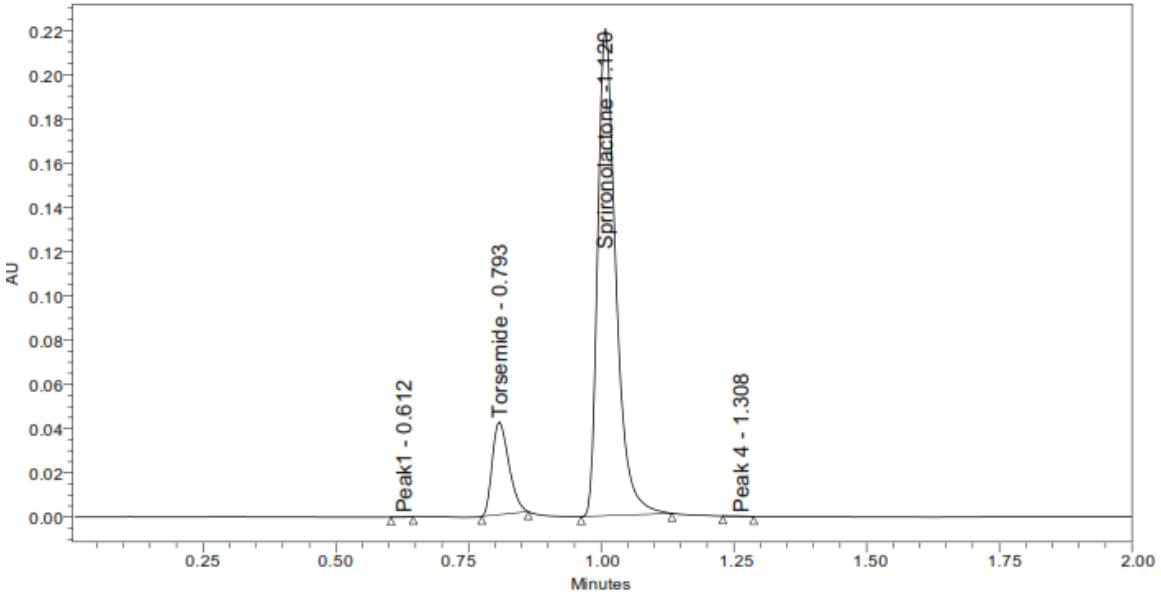

Fig.9: Chromatogram showing Peroxide

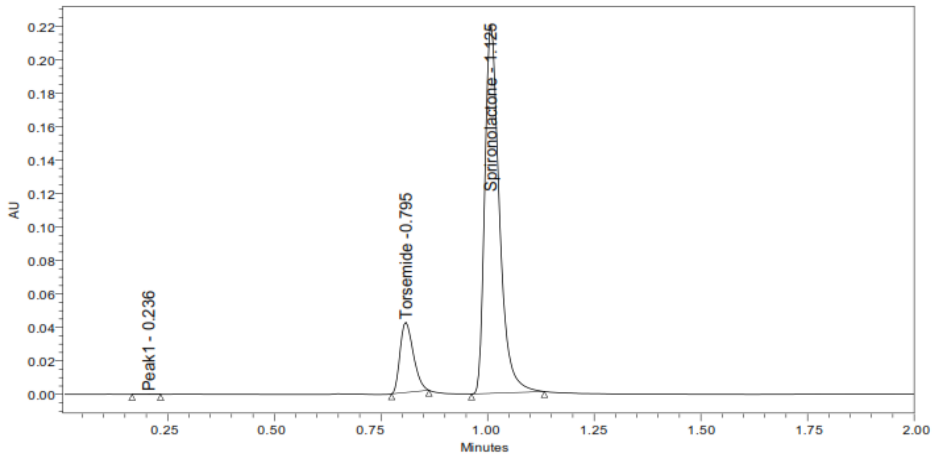

Fig.10: Chromatogram showing Thermal

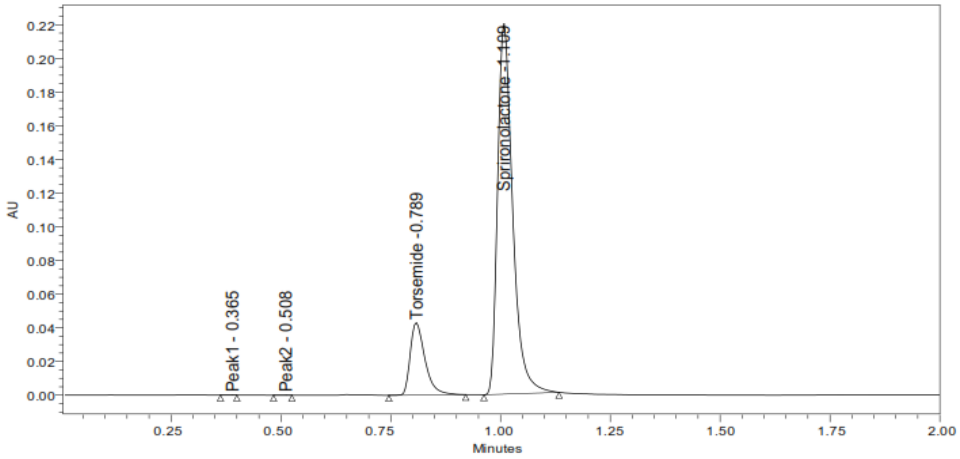

Fig. 11: Chromatogram showing Photo

Table 3: Degradation results

Sample Name	Torsemide	
	Area	% Degraded
Standard	107223	
Acid	98959	7.71
Base	98921	7.74
Peroxide	98978	7.69
Thermal	98851	7.81
Photo	98789	7.87

Table 4: Degradation results for Spironolactone

Sample Name	Spironolactone	
	Area	% Degraded
Standard	191642	
Acid	183252	4.38
Base	183532	4.23
Peroxide	183253	4.38
Thermal	187552	2.13
Photo	186452	2.71

Summary
The estimation of Torsemide and Spironolactone was done by RP-UPLC. The assay of Torsemide and Spironolactone was performed with tablets and the % assay was found to be 99.47 and 100.02 which shows that the method is useful for routine analysis. The linearity of Torsemide and Spironolactone was found to be linear with a correlation coefficient of 0.998 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision are %RSD should be not more than 2.0% and the method show precision of 0.1 and 0.7 for Torsemideand spironolactone which shows that the method is precise. The acceptance criteria of intermediate precision are RSD should be not more than 2.0% and the method show precision of 0.2 and 0.2 for Torsemide and Spironolactone which shows that the method is repeatable when performed on different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 99.74% and 99.40% for Torsemide and Spironolactone. The validation of the developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ are 3 and 10. The LOD and LOQ for Torsemide were found to be 2.98 and 10.00 and LOD and LOQ for Spironolactone were found to be 3.00 and 9.98. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method has good system suitability and precision under a given set of conditions.

Conclusion
Development and validation of RP-UPLC method for the simultaneous estimation of Torsemide and Spironolactone Pharmaceutical dosage forms". From the experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation Torsemide and Spironolactone was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Torsemide and Spironolactone in pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Torsemide and Spironolactone in pure and its pharmaceutical dosage forms.

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Conflict of Interest: Authors does not have interest of conflict.

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