3.1. DRUG PROFILE

3.1.1. Torsemide

Torsemide (TOR) is a pyridine-sulfonyl urea derivative. Chemically TOR is known to be 3-[4-[(3-methylphenyl) amino] pyridin-3-yl] sulfonyl-1-propan-2-ylurea [1-4]. It is highly useful in curing fluid retention (edema) coupled with congestive heart failure [5]. TOR finds immense application in the treatment of high blood pressure. It belongs to loop diuretics category. TOR exhibits direct attack on kidneys there by increasing the flow of urine. It is advisable in cirrhosis [6-9] management. A single dose of 2.5 to 5mg of TOR daily [10] is capable of lowering hypertension adequately without inducing diuresis [11]. Successful results are obtained when TOR is used to treat renal diseases [12]. On the other side continuous usage of TOR over a long period of time damages blood vessels of kidneys, heart and brain and finally leading to failure of kidneys, heart and brain respectively. Diarrhoea, blood vomiting, nausea, chest pain, headache and dizziness are the general side effects associated with the usage of TOR. TOR shows more prolonged diuretic effect and proportionately reduced potassium loss in comparison with the other diuretics [13]. In particular, when TOR is compared with furosemide (FUR) [14] it exhibits superior effects especially in heart failure cases. At the same time, one should not forget that TOR is only capable of controlling edema and elevated blood pressure and it is incapable to cure them.

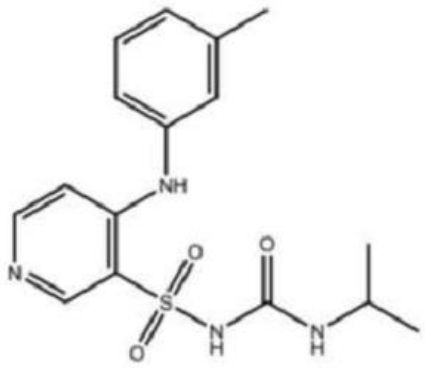


Figure.3.1. Structure of torsemide

IUPAC name: N-[(isopropyl amino) carbonyl]-4-[(3-methyl phenyl) amino] pyridine-3-sulfonamide.

Molecular formula: $C_{16}H_{20}N_4O_3S$

Molecular weight: 348.4

Melting point: 163-164^{0}C

Solubility: Soluble in water.

Brand name: Demadex, Diuver and Examide.

Administration: Oral

3.1.2. Spironolactone

Spironolactone (SPI) is 7α-acetylthio-3-oxo-17α-pregn-4-ene-21, 17-carbolactone [15]. It is a member of potassium-sparing diuretics. An important role is played by SPI in maintaining potassium levels in a body. SPI inhibits the action of aldosterone (a hormone secreted by the adrenal gland) which results in excretion of salt and fluid in the urine while potassium is retained. Generally SPI is used in treating hypertension, kidney disorder [16], edema, nephritic syndrome and cirrhosis. Hypokalaemia [17-22] can be avoided by the use of SPI. The most common side effects are loss of appetite, muscle pain, skin rash, breast swelling, diarrhoea, dizziness, leg cramps and tumors. In the laboratory animals it has been found that SPI caused tumors so it is better to disclose both risks and benefits associated with SPI medication. Both TOR and SPI in combination with other drugs can be recommended for the treatment of elevated blood pressure.

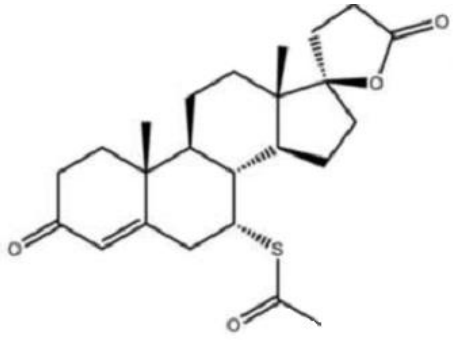


Figure.3.2. Structure of spironolactone

IUPAC name: [(7R,8R,9S,10R,13S,14S,17R)-10,13-dimethyl-3,5'-dioxo-1,2,3,4',5',6,7, 8,9,10,11,12,13,14,15,16 – hexadecahydro -3'H spiro [cyclopenta [a] phenanthrene -17, 2'-furan] -7- yl] ethanethioate.

Molecular formula: $C_{24}H_{32}O_4S$

Molecular weight: 416.6

Melting point: 134.5^0C

Solubility: Soluble in water, methanol, ethanol and chloroform.

Brand name: Aldactone.

Administration: Oral

Table.3.1: List of brand names of combined formulations of torsemide and spironolactone

S.No	Brand name	Available strength		Formulation	Manufacturer
1	DYNAMIDE PLUS	Torsemide Spironolactone	20mg	Tablet	Macleods Pharmaceuticals Pvt Ltd.
2	SPILACTONE T	Torsemide Spironolactone		Tablet	Sun Pharmaceutical Industries Ltd.
3	TORLACTONE	Torsemide Spironolactone	10mg, 5mg	Tablet	Sun Pharmaceutical Industries Ltd
4	ZATOR PLUS	Torsemide Spironolactone		Tablet	Unichem Laboratories Ltd.
5	TORSID PLUS	Torsemide Spironolactone	20mg, 5mg	Tablet	Aristo Pharmaceuticals Pvt Ltd.

3.2. LITERATURE SURVEY

Numerous analytical methods have been proposed for the estimation of TOR and SPI individually, in the combined dosage forms and in combination with other drugs. Several methods are available for their determination in biological samples. These methods generally involve spectrophotometry[23-34], thin layer chromatography[35], high performance thin layer chromatography[36-38] and liquid chromatography[39-41]. In addition to these methods gas chromatography[42,43], high performance liquid chromatography[44-71] and ultra performance liquid chromatography[72] have been developed. Much work is not available on stability studies[73-86] of TOR and SPI in the tablet form.

Golher [23] et al reported three spectrophotometric methods to determine TOR and SPI simultaneously in tablet dosage form. Different techniques employed for the detection includes absorbance ratio method, first order derivative spectroscopy and area under curve (AUC) method. The absorbance maximum of TOR and SPI was detected at 288nm and 238nm respectively. Beer's law was satisfied by both the drugs in all the three methods at a concentration range of 0-25mcg/ml. Absorbance ratio method was proposed at an isobestic point of 255nm. Two wavelengths 315nm for TOR and 225nm for SPI were selected to avoid the interference of other drugs in the determination of both the drugs through first order derivative spectroscopy. In AUC method, the wavelengths range preferred for TOR and SPI were 290-294nm and 236-240nm respectively. All the three methods were found to be simple, accurate and reproducible.

Bhadja [27] et al developed a simple and precise dual wavelength spectroscopic method for the estimation of TOR and amiloride hydrochloride (AML HCl) simultaneously in the combined pharmaceutical dosage form in methanol. The method was established using dual wavelengths for both TOR and AML HCl. Wavelengths of

299.66nm and 323.21nm were chosen for TOR and for AML HCl were 276.11nm and 300.0nm. Linearity range reported in case of TOR was 4 - 24µg/ml and it was 4 - 14µg/ml in case of AML HCl.

Patel [31] et al illustrated two methods namely first order derivative spectroscopic method and absorbance ratio method for the estimation of FUR and SPI simultaneously in combined dosage form that were proved to be accurate and reproducible. The parameters in both the methods were tabulated in **Table.3.2** and **3.3** respectively.

Table.3.2. Results of first order derivative spectroscopic method of SPI and FUR

S.No	Parameter	Spironolactone	Furosemide
1	Wavelength	25.0.80nm	350nm
2	Linearity range	5-25µg/ml	2-10µg/ml
3	% Recovery	100.88-101.46 %	98.25-100.00 %
4	% Assay for commercial formulation	98.8-100.9 %	98.25-102 %

Table.3.3. Results of absorbance ratio method of spironolactone and furosemide

S.No	Parameter	Spironolactone	Furosemide
1	Linearity range	5-25µg/ml	2-10µg/ml
2	% Recovery	98.8-100.55 %	99.24-102 %

Sharma [35] et al were succeeded in developing and validating a high performance thin layer chromatographic method for the determination of TOR and SPI in dosage form. The stationary and mobile phases used were precoated silica gel 50 F254 and a mixture of ethyl acetate, acetone and acetic acid in 10.5: 4: 1.5(v/v) respectively. A spot was recognized at 269.0nm. Different parameters were examined by making use of ICH guidelines. Linearity range for TOR and SPI was spotted at 360-850ng/spot. The method was simple, precise, accurate, rapid and suitable.

Shukla [44] et al described a precise and accurate reverse-phase HPLC method for the analysis of TOR. This method utilized a µBondapak C18 column with acetonitrile and phosphate buffer in 70:30(v/v) as mobile phase at a pH of 2.4. Linearity was found to be good in the range of 50-100µg/ml. The chromatograms exhibited good resolution without the interference of impurities. The retention time and the coefficient of variation were found to be 6.00±0.20 min and 0.998 at calibration point respectively.

Dubey [48] et al proposed and validated a sensitive reverse phase HPLC method for the estimation of TOR and SPI simultaneously in the combined dosage form at 238nm which was established to be simple, precise and accurate. Shimadzu LC10 ATvp system equipped with a Luna C_{18} column was used for the detection. A mobile phase of methanol: aectonitrile: phosphate buffer in 60:20:20(v/v) at a pH of 3.5 was used for the complete analysis. At a concentration range of 5-25µg/ml it followed Beer's law. The validation approach was carried out basing on the ICH guidelines.

Maulik [53] et al derived an economical RP-HPLC method for the simultaneous determination of FUR and SPI in the combined tablet dosage form with inertsil C18 column. HPLC separation was attained at 236nm with the help of a mobile phase composed of methanol, water in the ratio of 70:30(v/v). The development work was carried out at a pH of 3.20±0.05 and at a flow rate of 1.0ml/min. Retention times of FUR and SPI were reported at 3.64min and 6.69min respectively. Linearity was established in the range of 10-60µg/ml for SPI and 25-150µg/ml for FUR. The validation studies were supported by recovery studies. The method was confirmed to be sensitive, rapid, accurate and precise.

Patel [55] et al interpreted and validated a liquid chromatography method to evaluate TOR and AML HCl in accordance with ICH guideline Q2. Gradient reverse phase technique was monitored on an eclipse Enable C18 (4.6mm X 250mm, 5µm) column. At a wavelength of 288nm, mobile phase of composition acetonitrile and

potassium phosphate buffer (40:60, v/v), pH of 3.0 and a flow rate of 1.0ml/min were employed throughout the analysis. The results were shown in **Table.3.4.** The method was accepted in terms of linearity, accuracy, precision, sensitivity, specificity, robustness, ruggedness, and stability.

Table.3.4. Results of liquid chromatographic method of TOR and amiloride HCl

S.No	Parameter	Torsemide	Amiloride HCl
1	Linearity	2-12µg/ml, r = 0.998	2-12µg/ml, r = 0.999
2	LOD	0.07	0.007
3	LOQ	0.02	0.02
4	Precision	RSD < 1.7%	RSD < 1.5%

Ghodke [58] et al concluded a simple RP-HPLC method for the determination of TOR in bulk dosage form and was done on zorbax C18 (250mm X 4.6mm, 5µm) column at 288nm. The mobile phase utilized was a mixture of methanol: phosphate buffer in 50:50 ratios by volume. Ortho phosphoric acid was used to regulate the pH at 3.5. The flow rate was monitored at 1.3ml/min. The retention time reported for TOR was at 6.0$\pm$0.2 and it exhibited linearity in the range of 10-30µg/ml. The obtained percentage recovery of TOR was 99.80%.

Jain [62] et al established HPLC and spectrophotometric methods for the quantification of TOR and SPI by strictly following ICH Q2B guidelines. The method was conducted on an isocratic reverse phase inertsil C18 (520mm X 4.60mm, 5µm) column containing methanol: water (80: 20, v/v) mobile phase. Couple of wavelengths were selected for both TOR (243 and 330.5nm) and SPI (210 and 268nm) during UV spectrophotometric determinations. Both the methods were proved to be accurate and precise.

Zhang [66] et al reported a sensitive HPLC coupled with electrospray ionization tandem mass spectrometry (ESI-MS) for quantitative estimation of TOR in human plasma samples. G1 Sciences Inertsil ODS-3 (100mm X 2.1mm, 5.0µm) column with methanol and ammonium formate in the ratio of 60:40 by volume as mobile phase were selected for the entire analysis. The proposed method showed a flow rate of 0.2ml/min and linearity from 1-2500ng/ml in human plasma. The method accuracy was found within the range of 94.05% to 103.86%.

Sarkate [86] et al developed a stability indicating HPLC method for the validation of TOR. Different stress conditions were applied on TOR as described in ICH guidelines at 270nm. Ace5-C18 (250x4.6mm) column with a mobile phase composed of acetonitrile and water in 60:40 (v/v) at flow rate of 1ml/min was used to resolve TOR from degradation products. TOR exhibited the linearity within the range of 0.5-30µg/ml. The proposed method represented good accuracy, precision and robustness.

3.3. EXPERIMENTAL

3.3.1. Chemicals and solvents

The reference samples and the working standard of TOR and SPI of API grade were obtained from Macleods Pharmaceuticals Pvt. Ltd, Mumbai. The pharmaceutical formulation (Tide Plus TOR 10mg and SPI 25mg) was procured from provincial market. Methanol, acetonitrile and water were purchased from Merck Specialties Pvt. Ltd, Mumbai, India. Perchloric acid and the remaining buffer solutions (AR grade) were also purchased from Merck Specialties Pvt. Ltd, Mumbai, India.

3.3.2. Preparation of standard stock solution

TOR and SPI in the pure form were used for the preparation of standard stock solutions individually. Accurately weighed 10mg of TOR and 25mg of SPI were transferred into 10ml volumetric flasks separately. Initially, 5ml of the methanol was added to each of the drug to dissolve them. These solutions were sonicated for about 15min to dissolve them completely and then diluted with required quantity of methanol to acquire the proposed volume. Proper volumes of these solutions were further diluted with mobile phase to get different concentrations especially 10-60µg/ml for TOR and 25-50µg/ml for SPI. Equal quantities of the TOR and SPI were mixed and the resultant solution was used for simultaneous analysis.

3.3.3. Preparation of sample solution

Sample solution was prepared from finely ground uniform size powder of twenty tablets. Into a 10ml volumetric flask accurately weighed 10mg of TOR was quantitatively transferred and then 5ml of the methanol was poured. The solution was kept under sonication for about 15min. The flask was filled up to the mark by making use of mobile phase. Filtered solution was properly diluted with the mobile phase to acquire a concentration of 40µg/ml of TOR. Simultaneously a concentration of 100µg/ml of SPI was achieved as claimed on the label.

3.4. METHOD DEVELOPMENT

3.4.1. Detection of wavelength

The spectrum of diluted solutions of the two active ingredients TOR and SPI in methanol was recorded individually. The absorption spectrum of TOR and SPI was scanned on spectrophotometer in UV region i.e. 200-400nm. At a wavelength of 235nm the two drugs showed maximum overlapping and hence the entire determination was carried out at 235nm.

3.4.2. Choice of stationary phase

Initial trials were conducted by the use of octadecyl columns with different types, configurations and from different manufactures. The standard solution was injected into each of the columns and the peak area response was compared. Finally, kromasil RP-C18 column was found to be suitable for the simultaneous analysis of TOR and SPI.

3.4.3. Selection of mobile phase

Different mobile phases under isocratic conditions were tested in terms of sharp peak and base line separation. System suitability conditions in each case were examined. A mixture of methanol (MeOH), acetonitrile (ACN) and water in 50: 30: 20(v/v) was recognized as an appropriate mobile phase. This mobile phase exhibited high resolution, good base line separation and column efficiency but theoretical plates were found to be less. Theoretical plate count was improved when the pH of the mobile phase was adjusted to 6.8.

3.4.4. Flow rate

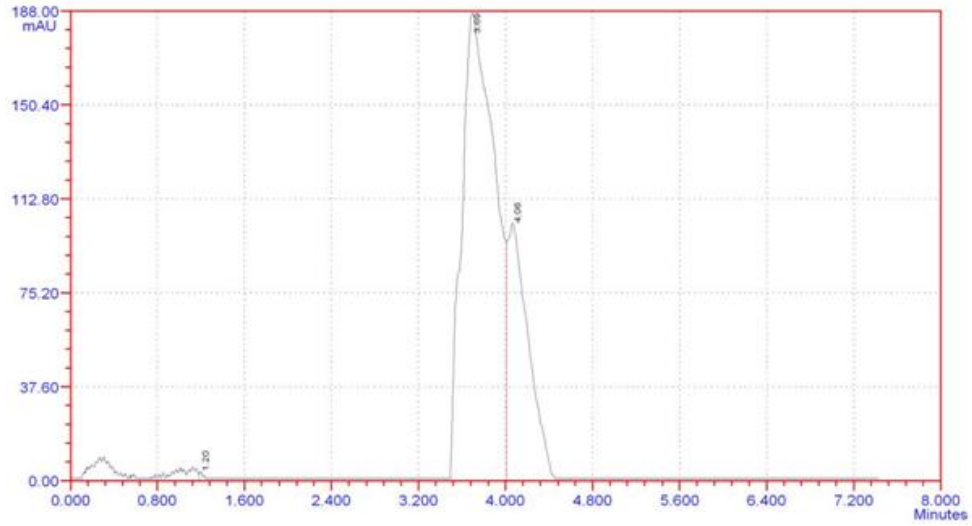
To attain optimum separation, mobile phase flow rates from 0.5 – 2ml/min were tested. Utmost saving of the solvents was achieved when minimum flow rate and minimum run time were maintained. The practice of flow rate at 1.0ml/min gave an extraordinary elution of the analytes.

3.4.5. Optimized chromatographic conditions

Several trials were conducted for the selection of optimum chromatographic conditions. Trial conditions were given in **Table.3.5** and the trial chromatograms were shown from **Figure.3.3.** to **3.8**. After several systematic trials, a simple, accurate and precise isocratic RP-HPLC method was established for the assay of TOR and SPI in pharmaceutical dosage forms. Optimized chromatographic conditions were presented in **Table.3.6** and the chromatograms of blank, TOR single, SPI single, standard and formulation were shown in **Figure 3.9, 3.10, 3.11, 3.12** and **3.13** respectively.

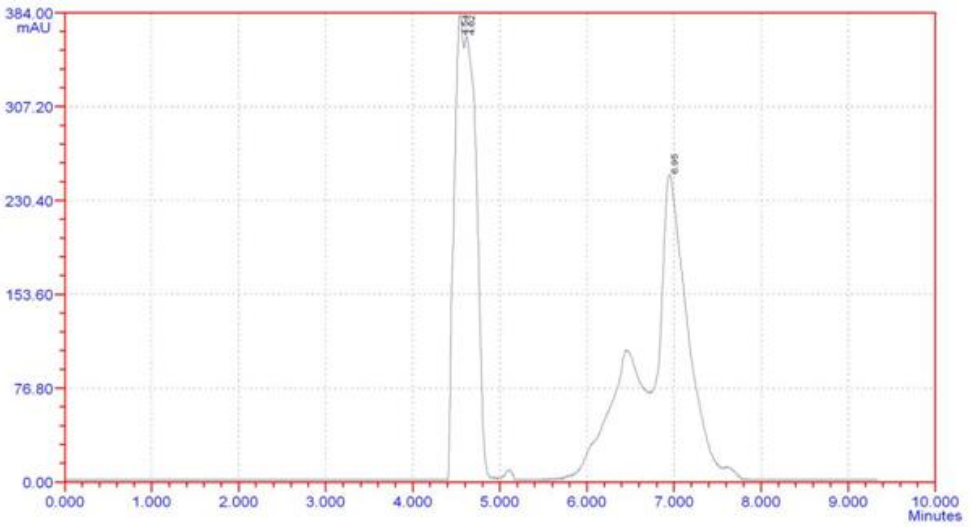
Table.3.5. Trial conditions of torsemide and spironolactone

Trial	Mobile phase (v/v)	Wavelength	pH of mobile phase	Column	Flow rate
I	MeOH: Water 50:50	235nm	5.3	Zodiac RP- C18	1.0ml/min
II	MeOH: Water 50:50	235nm	5.3	Kromasil RP- C18	1.0ml/min
III	MeOH: Water: ACN 40:30:30	235nm	5.3	Kromasil RP- C18	1.0ml/min
IV	MeOH: Water: ACN 50:30:20	235nm	5.3	Kromasil RP- C18	1.0ml/min
V	MeOH: Water: ACN 50:30:20	235nm	6.2	Kromasil RP- C18	1.0ml/min
VI	MeOH: Water: ACN 50:30:20	235nm	6.8	Kromasil RP- C18	1.0ml/min



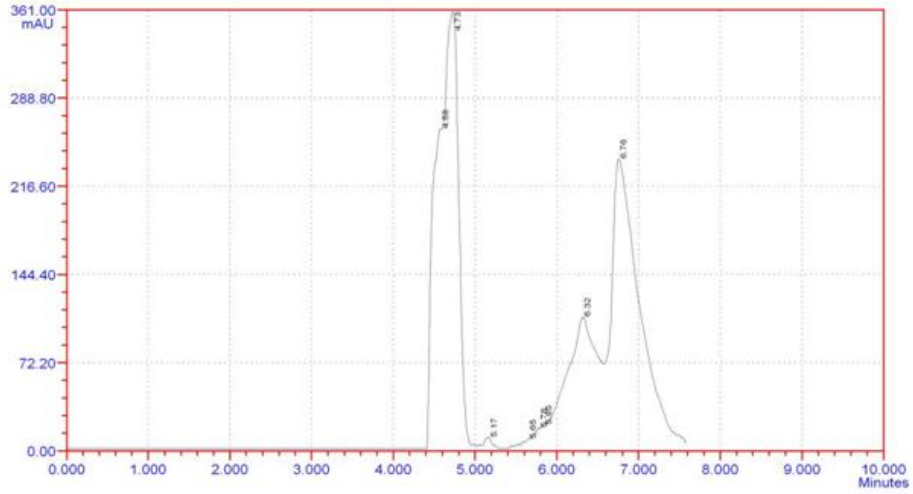
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		1.197	1042	3961.6	0.638	2.34	1976
2		3.694	20242	442589.0	71.225	1.20	569
3		4.064	11639	174847.7	28.138	4.90	1458
	Sum:		32923	621398.3	100.0000		

Figure.3.3. Trial chromatogram I



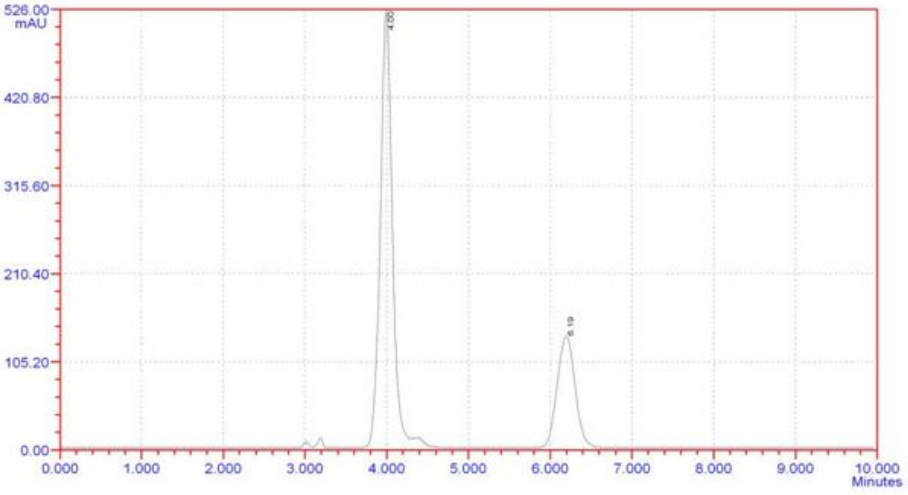
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.542	38463	289150.0	24.329	0.67	7275
2		4.617	36598	354626.5	29.839	5.74	4525
3		6.954	25437	544699.2	45.832	1.82	2102
	Sum:		100498	1188475.7	100.0000		

Figure.3.4. Trial chromatogram II



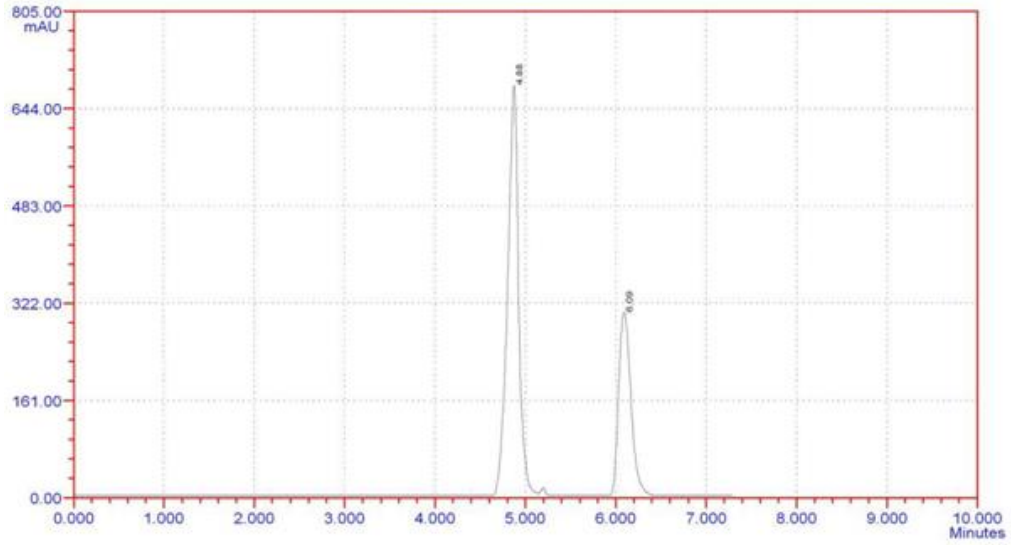
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.582	26418	211412.0	14.250	0.53	6533
2		4.728	36052	445461.4	30.027	1.33	2919
3		5.172	936	5358.6	0.361	0.81	16264
4		5.653	807	2655.6	0.179	0.76	58825
5		5.783	1663	9318.0	0.628	0.59	21233
6		5.847	1851	7058.3	0.476	0.78	46853
7		6.320	10452	275989.4	18.603	0.79	1142
8		6.758	23172	526303.8	35.476	2.48	1765
	Sum:		101351	1483557.3	100.0000		

Figure.3.5. Trial chromatogram III



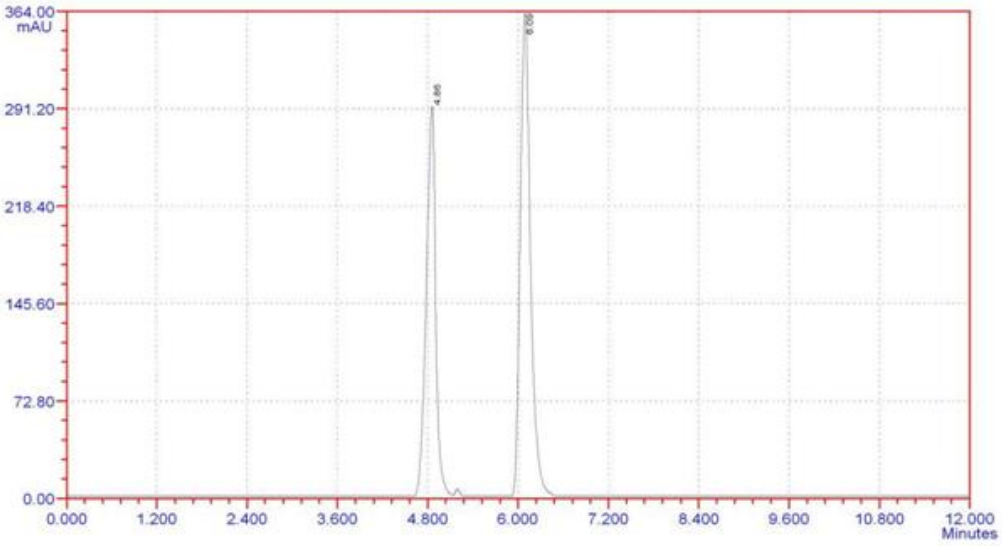
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.995	52742	554360.4	71.692	0.99	2879
2		6.193	13718	218897.0	28.308	1.07	3003
	Sum:		66460	773257.5	100.0000		

Figure.3.6. Trial chromatogram IV



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.875	62221	358491.9	35.314	0.60	1426
2		6.090	80322	656653.6	64.686	0.58	1106
	Sum:		142543	1015145.5	100.0000		

Figure.3.7. Trial chromatogram V



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.859	50340	341071.6	39.388	0.79	10249
2		6.092	67043	524858.9	60.612	1.15	12069
	Sum:		117383	865930.5	100.0000		

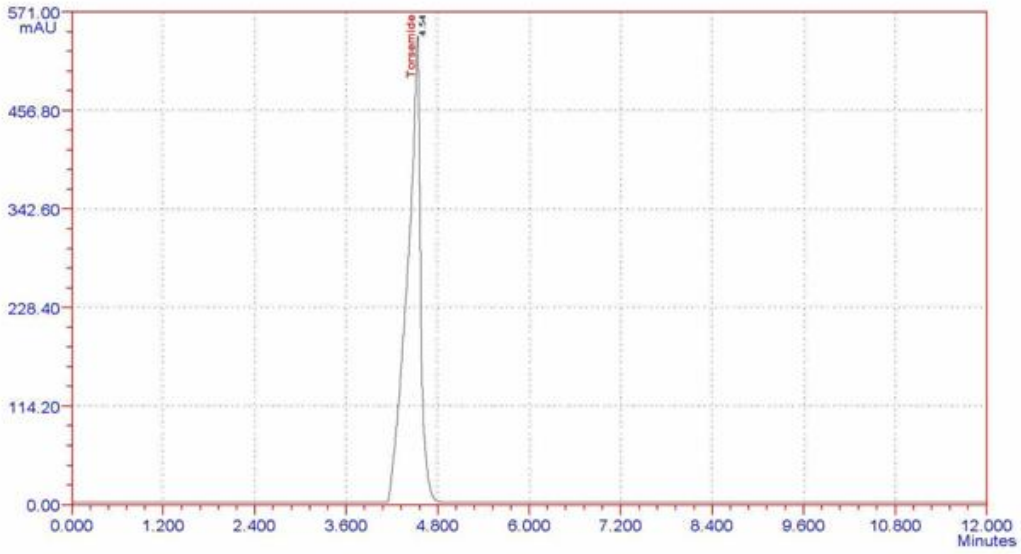
Figure.3.8. Trial chromatogram VI

Table.3.6. Optimized chromatographic conditions of torsemide and spironolactone

1	Pump mode	Isocratic	
2	Column	C 18 (250X4.6 mm, 5µm)	
3	Injector	Rheodyne	
4	Injector Volume	20µl	
5	Diluent	Methanol	
6	Mobile phase	Methanol: Acetonitrile: Water in 50: 30: 20 (v/v)	
7	Pump pressure	11.2 $\pm$ 7Mpa	
8	Mobile phase pH	6.8	
9	Wavelength	235nm	
10	Flow rate	1.0ml/min	
11	Run Time	12min	
12	Standard Concentration	Torsemide	40µg/ml
		Spironolactone	100µg/ml
13	Retention Time	Torsemide	4.55min
		Spironolactone	5.95min
14	Peak Area	Torsemide	451609
		Spironolactone	956848
15	Theoretical Plates	Torsemide	4311
		Spironolactone	6789
16	Tailing Factor	Torsemide	0.68
		Spironolactone	0.94

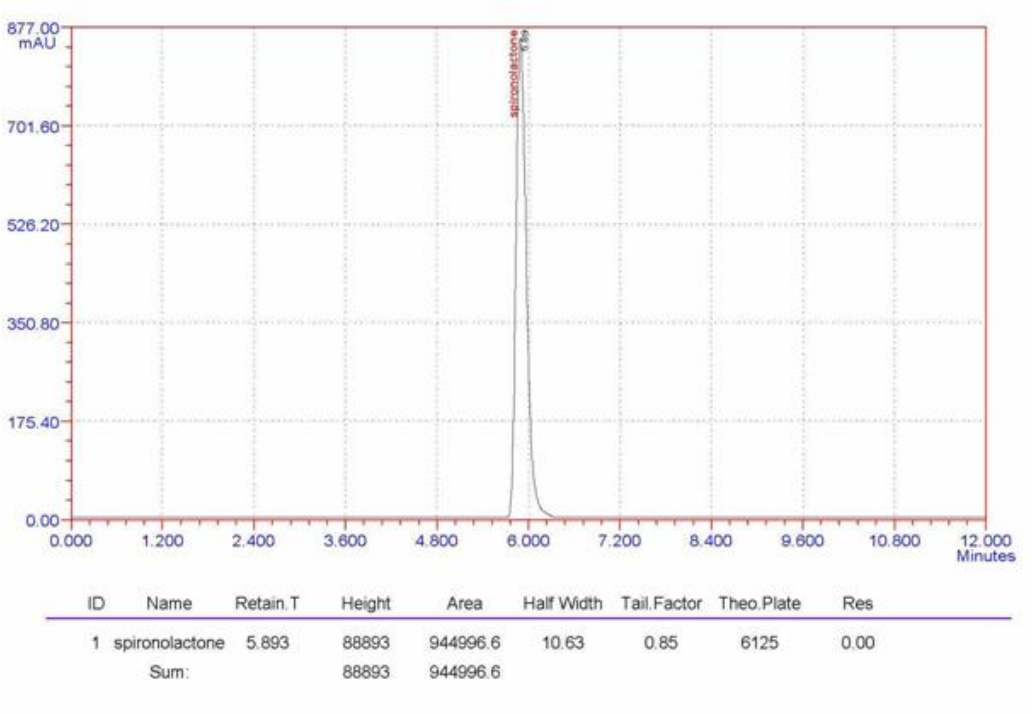
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
	Sum:		0	0.0				

Figure.3.9. Chromatogram of blank of torsemide and spironolactone



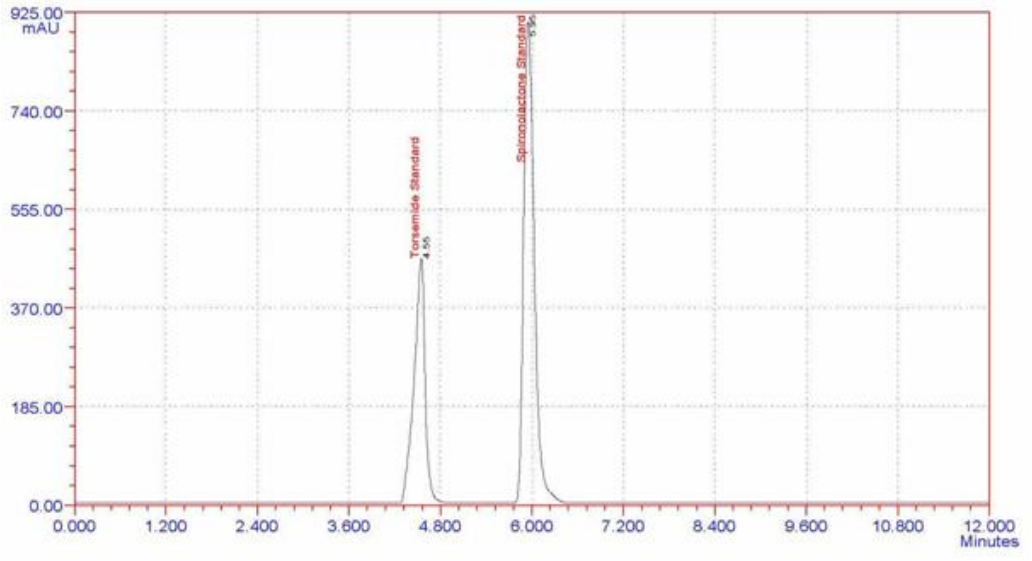
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1	Torsemide	4.537	48887	490189.6	10.03	0.67	4080	0.00
2		5.908	89757	951570.3	10.60	0.79	6190	4.69
	Sum:		138644	1441759.8				

Figure.3.10. Chromatogram of torsemide single



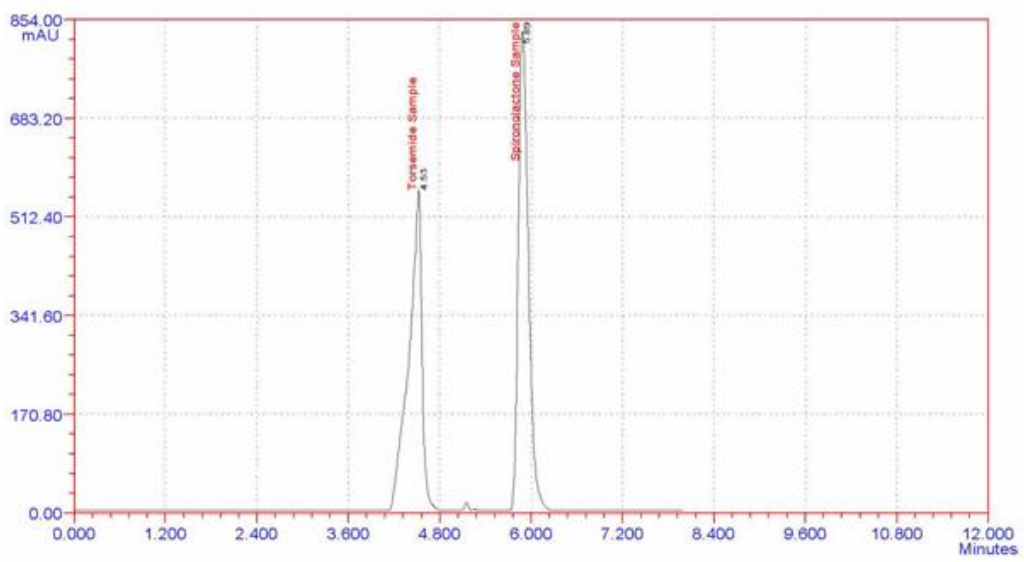
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1	spironolactone	5.893	88893	944996.6	10.63	0.85	6125	0.00
	Sum:		88893	944996.6				

Figure.3.11. Chromatogram of spironolactone single



ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1	Torsemide Standard	4.550	46162	451609.8	9.78	0.68	4311	0.00
2	Spironolactone Standard	5.950	93860	956848.3	10.19	0.94	6789	4.95
	Sum:		140022	1408458.1				

Figure.3.12. Chromatogram of torsemide and spironolactone standard



ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1	Torsemide Sample	4.525	45197	443694.9	9.82	0.63	4235	0.00
2	Spironolactone Sample	5.885	86525	955798.9	11.05	0.98	5658	4.60
	Sum:		131722	1399493.7				

Figure.3.13. Chromatogram of torsemide and spironolactone formulation

3.5. METHOD VALIDATION

The parameters of the proposed method were validated as described in ICH guidelines.

3.5.1. Specificity

Chromatograms of blank, standard and sample were compared to measure the specificity of the proposed method. The chromatograms of standard and formulation of torsemide and spironolactone confirms that there was no interference of diluents and placebo in the analysis. Hence the method was found to be specific. Symmetrical peaks were observed in the standard solution with the retention times of 4.55min for torsemide and 5.95min for spironolactone.

3.5.2. System suitability

Freshly prepared standard stock solutions of torsemide and spironolactone were used to evaluate system suitability conditions. Equal volumes of standard concentration of torsemide and spironolactone were mixed well in another volumetric flask. From this solution, 20µl of the sample was injected into HPLC system. The results obtained were given in **Table.3.7** to express the system suitability of the proposed method.

Table.3.7. Results of system suitability of torsemide and spironolactone

S.No	Parameter	Torsemide	Spironolactone
1	API concentration	40µg/ml	100µg/ml
2	Retention Time	4.55min	5.95min
3	Resolution	……	4.95
4	Peak Area	451609	956848
5	Theoretical Plates	4311	6789
6	Tailing Factor	0.68	0.94

3.5.3. Linearity

A series of six different concentration levels were prepared to determine the linearity of the method. Calibration plots of concentration against peak area were constructed separately for both TOR and SPI to evaluate linearity. Regression of the plots was calculated by the use of least square method. The correlation coefficients (r^2) for both TOR and SPI were obtained to be 0.999.The results of linearity were represented in **Table.3.8** and the plots were presented in **Figure.3.14** and **3.15**.

Table.3.8. Results of linearity of torsemide and spironolactone

S.No	Torsemide		Spironolactone	
	Concentration in µg/ml	**Peak Area**	**Concentration in µg/ml**	**Peak Area**
1	10	193642	25	220513
2	20	284529	50	500819
3	30	355954	75	715681
4	40	451609	100	956848
5	50	531253	125	1225941
6	60	620529	150	1451013

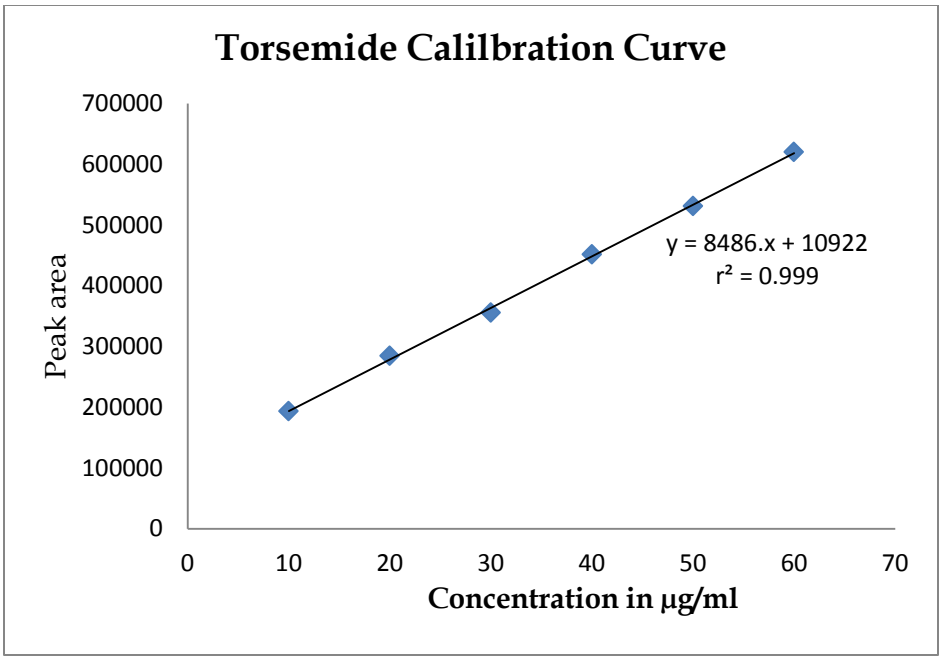


Figure.3.14. Calibration curve of torsemide

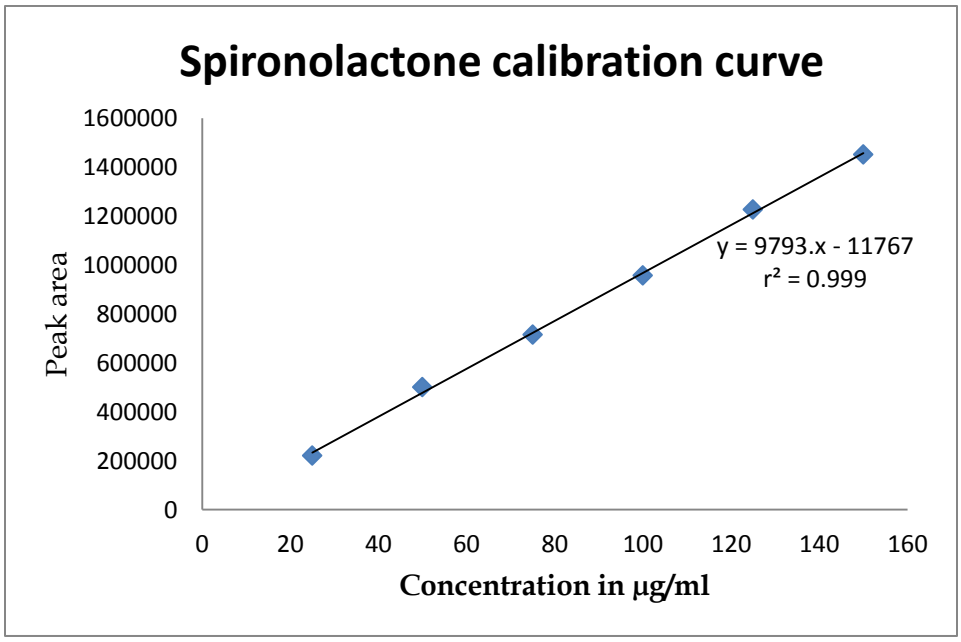


Figure.3.15. Calibration curve of spironolactone

3.5.4. Accuracy

Standard addition method was employed to measure the accuracy of the suggested method. Three concentration levels (50%, 100% and 150%) were spiked with the reference solution and the measurements were made in triplicate at each level. Percentage RSD was used to evaluate the recovery of the two drugs TOR and SPI and the results were given in **Table.3.9** and **3.10** respectively. Comfortable recoveries ranging from 99.68 to 101.79 for torsemide and from 98.28 to 101.81 for spironolactone were attained.

3.5.5. Precision

The degree of repeatability of an analytical method under ordinary conditions was termed as precision. Measurement of precision depends on both intra-day and inter-day precisions. In case of intra-day precision six replicate standard solutions of TOR and SPI were injected and the percentage RSD was calculated. In case of inter-day precision six replicate standard solutions of TOR and SPI were injected on three consecutive days and percentage RSD was found to be 1.18 and 0.17 for TOR and SPI respectively. The results of intra-day precision and inter-day precision were furnished in **Table.3.11** and **3.12** respectively. Good repeatability of the method was confirmed from the results of both intra-day and inter-day precisions.

3.5.6. Ruggedness

Ruggedness is the measure of reproducibility of the proposed method. Ruggedness was estimated by different analyst using different columns on different days. Six dilutions were tested and the results were shown in **Table.3.13.** From the reported data, it was found that the percentage RSD was within the suitable criteria. Thus, the proposed method was confirmed to be reproducible.

Table.3.9. Recovery results of torsemide

S.No	Spiked Level	Concentration in µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	20	10	30	29.909	99.697
2		20	10	30	29.906	99.688
3		20	10	30	30.538	101.792
4	100%	20	20	40	40.452	101.131
5		20	20	40	39.964	99.909
6		20	20	40	40.691	101.728
7	150%	20	30	50	50.635	101.271
8		20	30	50	50.273	100.547
9		20	30	50	50.259	100.518

Table.3.10. Recovery results of spironolactone

S.No	Spiked Level	Concentration in µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	50	25	75	76.208	101.611
2		50	25	75	74.642	99.523
3		50	25	75	74.669	99.558
4	100%	50	50	100	99.380	99.380
5		50	50	100	98.282	98.282
6		50	50	100	98.965	98.965
7	150%	50	75	125	124.601	99.681
8		50	75	125	127.264	101.811
9		50	75	125	125.843	100.675

Table.3.11. Results of intra-day precision of torsemide and spironolactone

S. No	Torsemide at 40µg/ml	Spironolactone at 100µg/ml
1	461899	958110
2	449174	954289
3	451396	942871
4	447213	958193
5	457383	954345
6	451002	951570
% RSD	1.221	0.595

Table.3.12. Results of inter-day precision of torsemide and spironolactone

S. No	Torsemide at 40µg/ml	Spiranolactone at 100µg/ml
1	454481	949137
2	457619	946376
3	459512	945815
4	453163	949813
5	444183	948269
6	451563	949312
% RSD	1.186	0.174

Table.3.13. Results of ruggedness of torsemide and spironolactone

S. No	Torsemide at 40µg/ml	Spironolactone at 100µg/ml
1	462677	942332
2	452682	953904
3	467724	954919
4	446336	951134
5	451002	944058
6	466559	955601
%RSD	1.96	0.60

3.5.7. Robustness

Small deliberate changes were introduced with respect to wavelength, pH and mobile phase to measure the robustness of the method. The effect of these changes on chromatographic parameters was observed. In particular, retention time, tailing factor and number of theoretical plates were compared with the standard solution. Percentage change in the results was calculated and was found to be within the agreeable limit. Hence the method is considered to be valid in the given limits. Robustness results were furnished in **Table.3.14.**

3.5.8. Limit of detection and limit of quantification

Limit of detection enables to measure detectable response at the lowest possible concentration. On the other hand limit of quantification requires the measurement of quantified response with enough accuracy and precision at a minimum concentration level. Solutions of different concentrations were prepared and all the solutions were investigated repeatedly to evaluate LOD and LOQ values. The resultant values were shown in **Table.3.15.**

Table.3.14. Results of robustness of torsemide and spironolctone

S.No	Condition	Change	Torsemide		Spironolactone	
			Area	% Change	Area	% Change
1	Standard	NO Change	451609	……..	956848	…….
2	MP 1	MeOH:ACN: Water (55:25:20)	458467	1.519	951527	0.556
3	MP 2	MeOH:ACN: Water (45:35:20)	450011	0.354	948278	0.896
4	WL 1	232nm	451337	0.06	956865	0.002
5	WL 2	238nm	456682	1.123	944990	1.239
6	pH 1	6.7	457582	1.323	950277	0.687
7	pH 2	6.6	449742	0.413	949833	0.733

Table.3.15. Results of LOD and LOQ of torsemide and spironolactone

Drug	LOD	LOQ
Torsemide	0.05µg/ml	0.16µg/ml
Spiranolactone	0.08µg/ml	0.26µg/ml

3.5.9. Solution stability

To measure the stability of torsemide and spironolactone, standard solution was prepared and was kept aside for about two days. At regular intervals of time, the solution was injected and the chromatographic parameters were compared with the freshly prepared standard solution. The results were presented in **Table.3.16.** The solution exhibited stability up to 36hr.

Table.3.16. Results of solution stability of torsemide and spironolactone

S.No	Time in Hours	Torsemide		Spironolactone	
		Area	% Assay	Area	% Assay
1	2.0	456472	101.077	943966	98.654
2	4.0	458976	101.631	958780	100.202
3	8.0	458434	101.511	950212	99.306
4	12.0	447066	98.994	944373	98.696
5	24.0	444728	98.476	944030	98.660
6	36.0	443794	98.269	954729	99.778
7	48.0	440669	97.577	931387	97.339

3.5.10. Formulation

Into the HPLC system, 20µl of freshly prepared sample was injected and the corresponding peak response was measured. The percentage assay was found to be 98.247 for TOR and 99.89 for SPI. Results were introduced in **Table.3.17**.

Table.3.17. Results of torsemide and spironolactone formulation

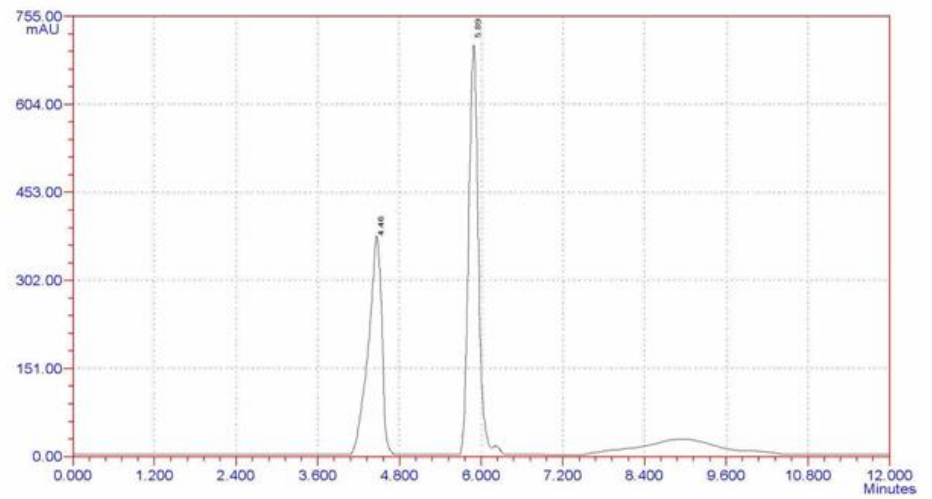
S.No	Drug	Brand	Dosage	Amount	Amount	%Assay
1	Torsemide	Tide Plus	10mg	40µg/ml	39.299µg/ml	98.247
2	Spiranolactone		25mg	100µg/ml	99.89µg/ml	99.89

3.6. FORCED DEGRADATION STUDIES

The stability of TOR and SPI formulation under different stress conditions were measured through degradation study. Degradation peaks under each stress condition were shown in **Table.3.18**. Corresponding degradation chromatograms were given from **Figure.3.16** to **Figure.3.22.**

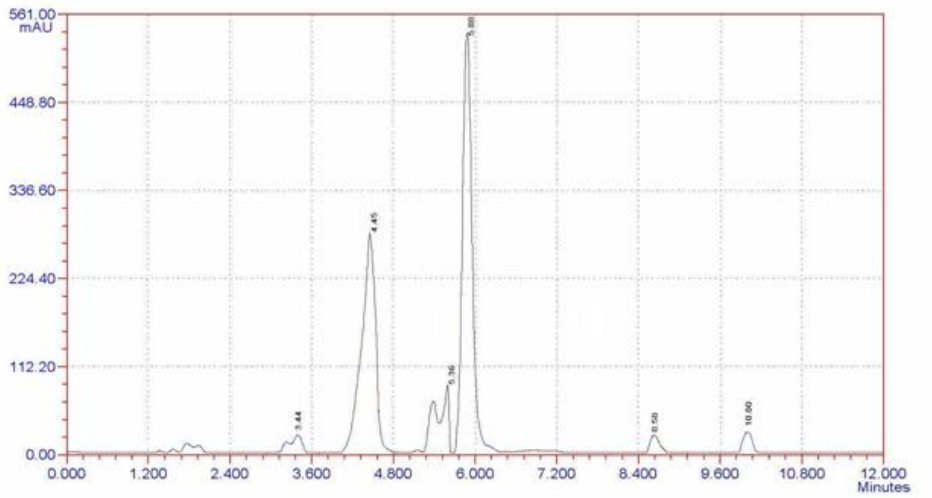
Table.3.18. Forced degradation studies of torsemide and spironolactone

S. No	Condition	No of degradation Peaks Observed
1	Aqueous	0
2	Acidic	4
3	Basic	1
4	Peroxide	4
5	Thermal	3
6	Sun Light	2
7	UV light	3



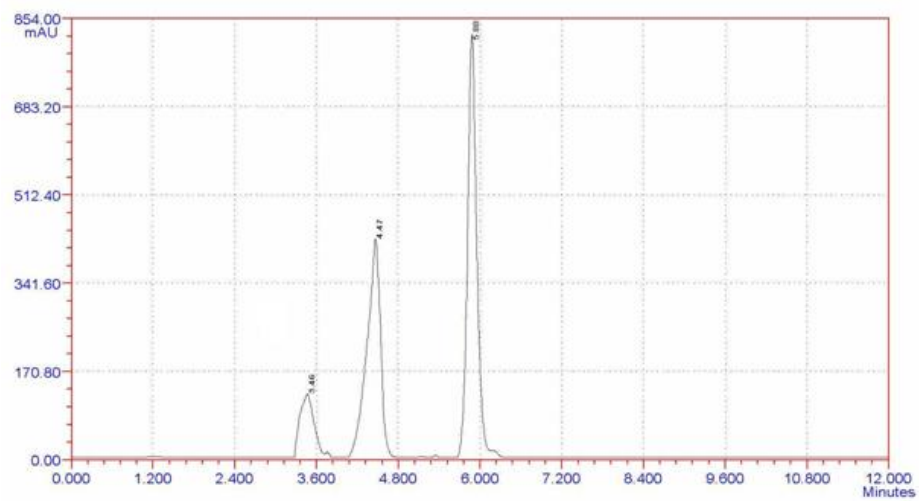
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		4.462	36797	439741.3	11.95	0.74	2779	0.00
2		5.892	82393	879365.9	10.67	0.66	6074	4.46
	Sum:		119190	1319107.2				

Figure.3.16. Chromatogram of aqueous degradation



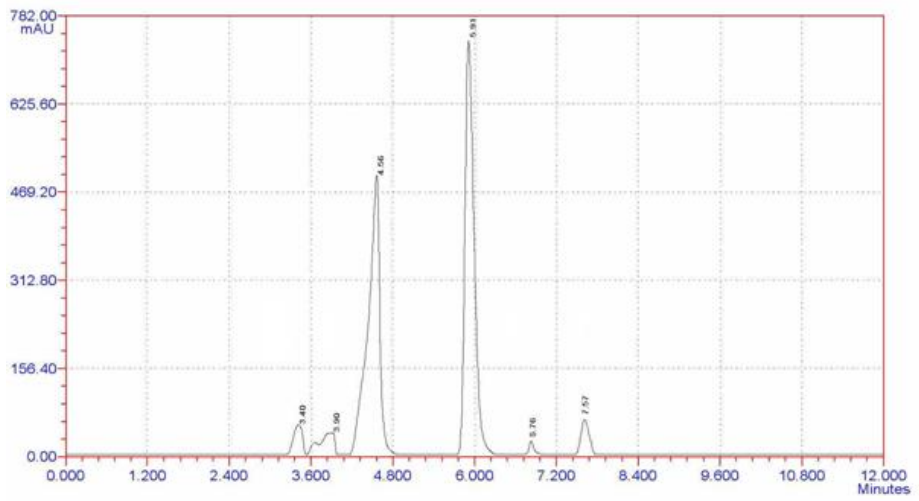
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		3.440	2396	84204.0	35.14	0.76	191	0.00
2		4.453	35919	408188.2	11.36	0.82	3061	1.54
3		5.365	1779	31307.5	17.60	0.56	1852	2.22
4		5.883	81878	862497.2	10.53	1.30	6217	1.30
5		8.582	1279	105670.8	82.62	2.83	215	2.04
6		10.002	585	98691.7	168.70	8.18	70	0.40
	Sum:		123836	1590559.4				

Figure.3.17. Chromatogram of acid degradation



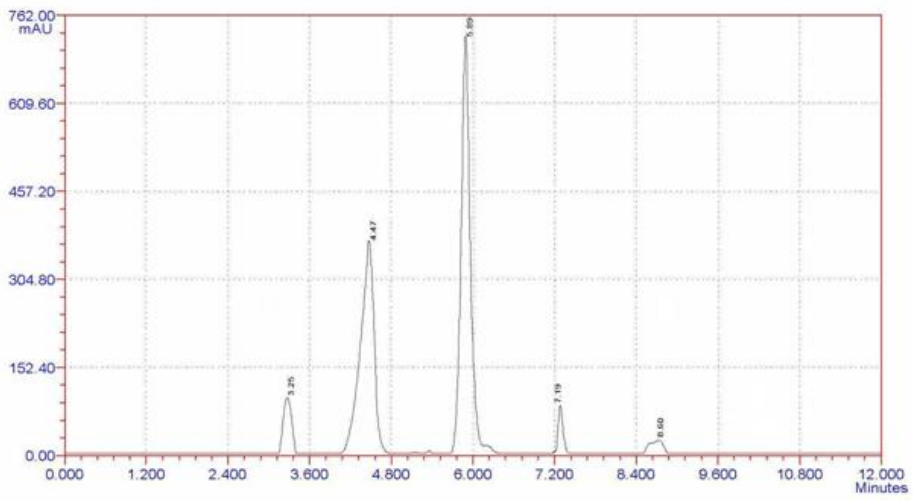
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		3.458	2234	85161.0	38.12	0.64	164	0.00
2		4.465	37146	415967.7	11.20	0.67	3169	1.44
3		5.883	85610	890712.5	10.40	0.65	6373	4.63
	Sum:		124990	1391841.2				

Figure.3.18. Chromatogram of base degradation



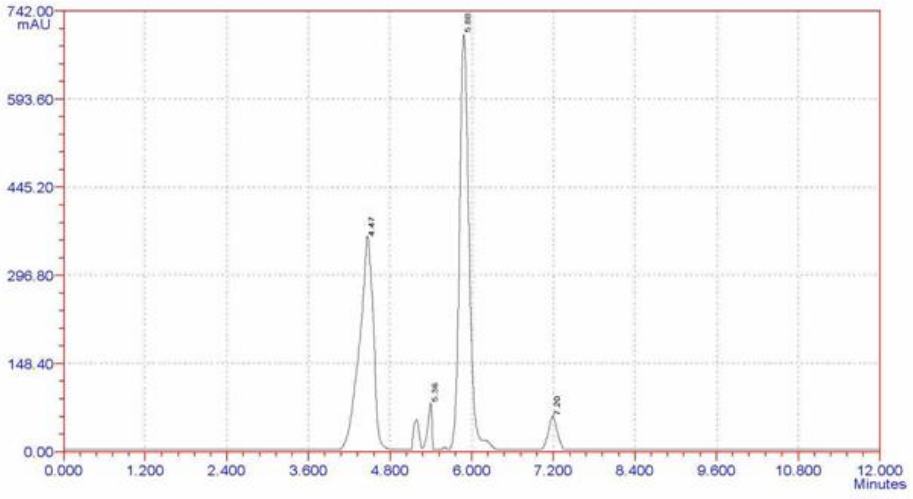
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		3.403	3786	39532.9	10.44	0.50	2117	0.00
2		3.905	2463	52399.3	21.27	0.68	671	1.12
3		4.563	49746	423835.2	8.52	0.63	5717	1.56
4		5.912	96304	799903.6	8.31	1.26	10096	5.66
5		6.763	2813	77355.1	27.50	0.84	1206	1.68
6		7.573	3475	29191.8	8.40	0.56	16199	1.59
	Sum:		158587	1422217.9				

Figure.3.19. Chromatogram of peroxide degradation



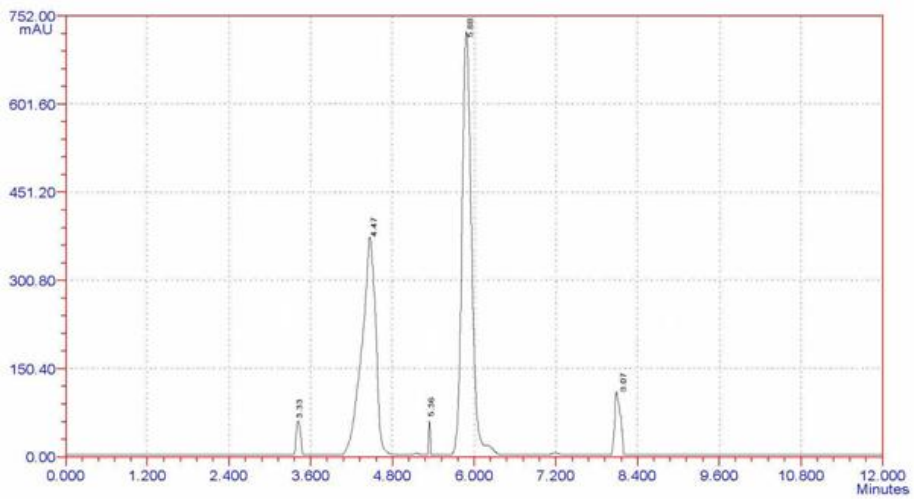
ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		3.250	3826	101020.0	26.40	0.50	302	0.00
2		4.470	35693	407846.0	11.43	0.78	3050	2.28
3		5.890	79845	702031.3	8.79	1.06	8944	4.96
4		7.185	2276	59297.4	26.05	1.07	1516	2.62
5		8.597	2659	37067.9	13.94	0.76	7580	2.49
	Sum:		124299	1307262.6				

Figure.3.20. Chromatogram of thermal degradation



ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		4.467	37083	424721.5	11.45	0.67	3031	0.00
2		5.365	1429	21426.2	14.99	0.56	2552	2.40
3		5.885	77410	690655.1	8.92	1.18	8671	1.53
4		7.195	2157	51931.9	24.08	0.85	1780	2.80
	Sum:		118079	1188734.6				

Figure.3.21. Chromatogram of light degradation



ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate	Res
1		3.332	4841	47252.4	9.76	0.50	2322	0.00
2		4.467	37083	413835.3	11.16	0.66	3193	3.83
3		5.365	1645	23006.5	13.99	0.59	2933	2.52
4		5.885	79940	736752.9	9.22	1.17	8126	1.58
5		8.067	3984	81145.5	20.37	0.65	3126	5.21
	Sum:		127493	1301992.6				

Figure.3.22. Chromatogram of UV light degradation

3.7. RESULTS AND DISCUSSION

The proposed method was planned to develop a stability indicating HPLC method for the simultaneous estimation of torsemide and spironolactone in commercial formulation. UV spectrophotometer was used to monitor the wavelength selection. Maximum absorption was exhibited by the both drugs at 235nm and thus the entire work was carried out at this wavelength only. Basic trials were conducted for the choice of column, stationary phase and mobile phase. Finally, successful results were obtained when the process utilized kromasil RP-C18 column with mobile phase of methanol, acetonitrile and water in 50: 30: 20 (v/v). The total analysis was carried out more comfortably at ambient temperature. Optimized chromatographic conditions were achieved only at a pH of 6.8. Orthophosphoric acid was an important factor in maintaining the constant pH. The results were tabulated in **Table.3.6.**

Method validation was started with earlier measurement of specificity. The chromatograms of blank (**Figure.3.9**) and standard (**Figure.3.12**) of torsemide and spironolactone were studied and it was concluded that excipients have no effect in the analysis. Various properties of chromatogram were studied especially tailing factor (<2.0), resolution (>2.0) and theoretical plates (> 2000) and were given in **Table.3.7.** The simple analytical conditions took very less time (<6 min) for both the drugs and facilitate the analysis of more number of samples in a less time. Linearity of torsemide was observed in the concentration range of 10-60µg/ml and corresponding regression equation was $y = 8486.x + 10922$ ($r^2 = 0.999$). Linearity of spironolactone was found in the range of 25-150µg/ml with regression equation of $y = 9793.x – 11767$ ($r^2 = 0.999$). Corresponding linearity results were presented in **Table.3.8.** Accuracy of the method was predicted from the recovery studies. The recovery studies were conducted at three spiked concentrations and the results (**Table.3.9 & 3.10**) were within the requisite range.

Sufficient number of aliquots of a homogeneous sample was injected to determine the precision of the method. Intra-day precision and inter-day precision measurements were used to check the precision of the method. The calculated percentage RSD for torsemide and spironolactone were in the agreeable range (<2). The results were furnished in **Table.3.11** and **3.12.** Ruggedness was performed by different analyst on three different days using six replicate injections. The results shown in **Table.3.13** were in comfortable range. Small changes were introduced in chromatographic parameters to analyze robustness. Results of robustness were found to be in suitable range and were introduced in **Table. 3.14**. The LOD and LOQ values were shown in **Table.3.15.** Stability study (**Table.3.16**) was conducted over a period of 48hr and it was reported that the solution was stable upto 36hr. The validated method was applied for the assay of commercial formulation of torsemide and spironolactone. The results can be viewed from the **Table.3.17.**

Different stress conditions were applied to the standard solution of torsemide and spironolactone to measure the stability of the molecule. Various stress conditions such as acidic, basic, aqueous, peroxide, thermal, light and UV light were applied for inducing stress in the molecule. The results were tabulated in **Table.3.18.** Thus, the developed stability indicating isocratic RP-HPLC method was found to be simple, accurate and precise.

3.8. CONCLUSIONS

The principal aim of the present investigation was stability indicating studies of torsemide and spironolactone in pharmaceutical formulations. The mobile phase used in the proposed method was simple and economical. Validation of the demonstrated method was done in terms of linearity, precision, ruggedness, robustness, sensitivity and solution stability. Sample recoveries recommended the non-interference of excipients and diluents during the analysis. The stability of the molecule was measured through forced degradation studies. Degradation chromatograms revealed the information about the number of degradation products formed under different stress conditions. Finally, a simple, accurate and precise stability indicating method for the simultaneous estimation of TOR and SPI was developed. Thus the illustrated method can be conveniently applied for the regular analysis of TOR and SPI in the pharmaceutical dosage forms.

Degradation studies are highly useful in investigating degradation path ways and degradation products. Hence the forced degradation studies provide valuable information that is highly beneficial during the manufacturing, formulation development and packing of TOR and SPI in the combined dosage forms. Thus future research must focus on evaluating degradation path ways, degradation products and mechanism of action of degradation products.

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