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A stability indicating LC method for simultaneous determination of torsemide, spironolactone and their related substances in tablet formulation

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ABSTRACT

An isocratic reverse phase liquid chromatography method has been developed for quantitative determination of Torsemide and Spironolactone along with their related compounds using a 150 X 4.6 mm, 5µ Hypersil BDS C8 column with a mobile phase composition of buffer pH 5.0: methanol in equal quanitites. The flow rate was 1.0 mL min^{-1} and wavelength was set at 260 nm. Resolution between torsemide and its impurity, and that due to Spironolactone and its impurity canrenone was more than 2.0 and 3.0 respectively. The method was validated for selectivity, linearity, accuracy, precision, limit of detection and limit of quantitation. Impurities of torsemide and spironolactone gave linear response. For the assay study torsemide and spironolactone showed linear response. The stress studies showed that the method was specific, selective to study torsemide, spironolactone and impurity1, impurity 2, impurity 3 of torsemide and impurity 4 of spironolactone. The peak purity of analyte showed that unknown degradation products formed during stress studies did not interfere with the determination of all the studied analytes. The mass balance for assay was achieved for torsemide and spironolactone.

KEYWORDS

Torsemide;
Spironolactone;
Canrenone;
Related substances;
Simultaneous determination.

INTRODUCTION

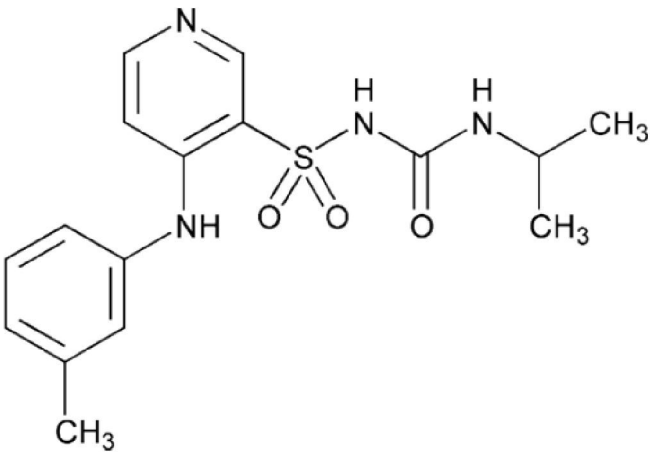
Spironolactone, 7α-(acetylsulfanyl)-3',4'-dihydrospiro [androst-4-ene-17,2'(5'*H*)-furan]-3,5'-dione (SL) is a synthetic steroid that acts as a competitive antagonist to aldosterone used clinically in conditions such as congestive heart failure, hepatic ascites, primary aldosteronisms and essential hypertension. Torsemide, 1-(1-Methylethyl)-3-[[4-[(3-methylphenyl) amino]pyridin-3-yl]sulphonyl]urea (TD) is a pyridine-sulfonyl urea type loop diuretic mainly used in the management of edema associated with congestive heart failure. It is also used at low doses for the management of hypertension.

The combination product of SL and TD is used for the treatment of congestive heart failure. Pharmaceutical impurities are the unwanted chemicals that remain with the APIs or develop during formulation, or upon

degradation of both API and formulated APIs to medicines. The presence of these unwanted chemicals even in small amounts might influence the efficacy and safety of pharmaceutical products. Determinations of drug impurity and drug degradation products are very important from both pharmacological and toxicological perspectives. The aim of the present study is to develop a stability indicating method for the determination of SL, TD along with related compounds. In this paper we describe validation of an assay and related substances method for accurate quantitation of TD and SL and their four related compounds (Imp 1: 1-ethyl-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulphonyl]urea; Imp 2: 4-[(3-methylphenyl)amino]pyridine-3-sulphonamide; imp 3: 1-butyl-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulphonyl]urea; imp 4: 17-hydroxy-3-oxo-17a-pregna-4,6-diene-21-carboxylic acid gamma-lactone) in tablet dosage form. Figure 1

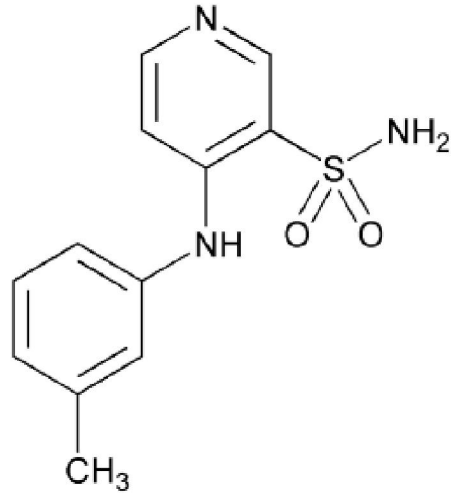
Many methods have been reported for determination of SL and TD along with other diuretics for screening of drugs in various matrices by various analytical techniques like in human urine by HPLC[1-3], LC-ESI-MS[4],and GC-MS after extractive methylation[5], fast LC-MS/MS[6] and in bovine milk by UPLC-tandem mass spectrometry[7], using micellar mobile phases[8,9].

For SL and impurities, a spectrophotometric method is reported by partial least square regression[10], HPLC and TLC methods are reported for SL and its degradation product[11] and its metabolites in human plasma[12,13]. Literature review showed solubilization and stability of SL solution studied in β-cyclodextrin derivatives[14] and Impurities of SL are isolated and studied[15,16]. For SL a HPLC method is reported for determination from formulation[17]. SL and chlorthalidone in combination is reported by HPLC[18].



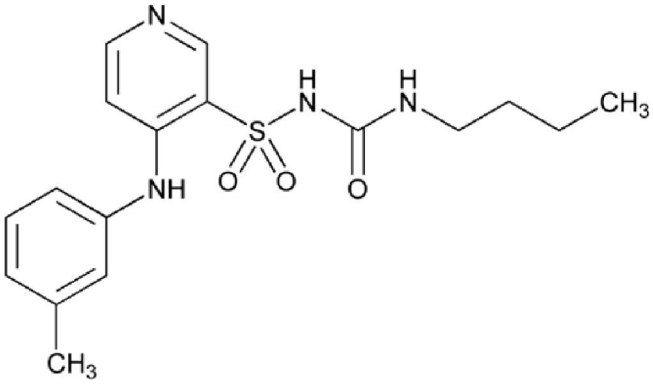
Torsemide (TD)

1-(1-Methylethyl)-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulphonyl]urea



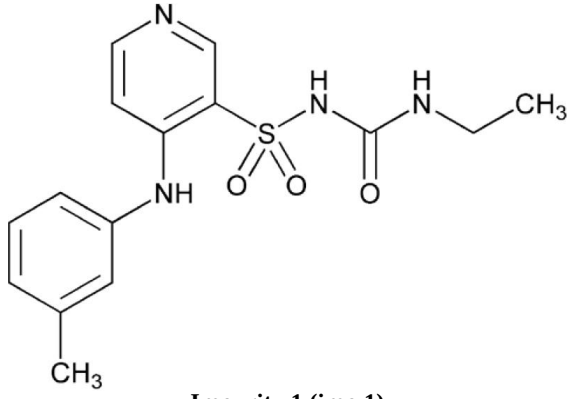
Impurity 2 (imp 2)

4-[(3-methylphenyl)amino]pyridine-3-sulphonamide



Impurity 3 (imp 3)

1-butyl-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulphonyl]urea.



Impurity 1 (imp 1)

1-ethyl-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulphonyl]urea

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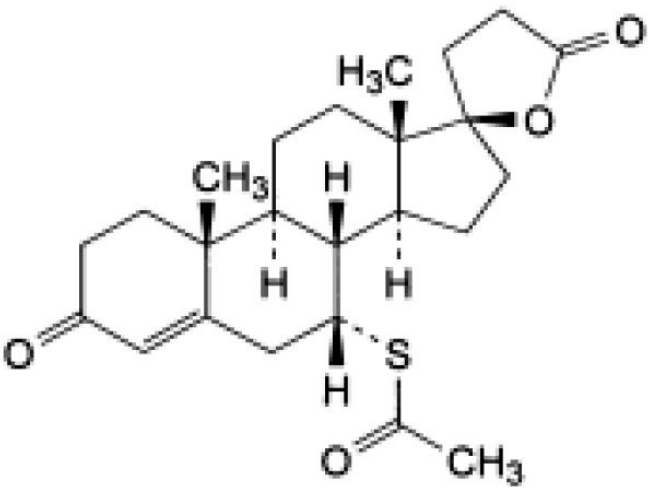
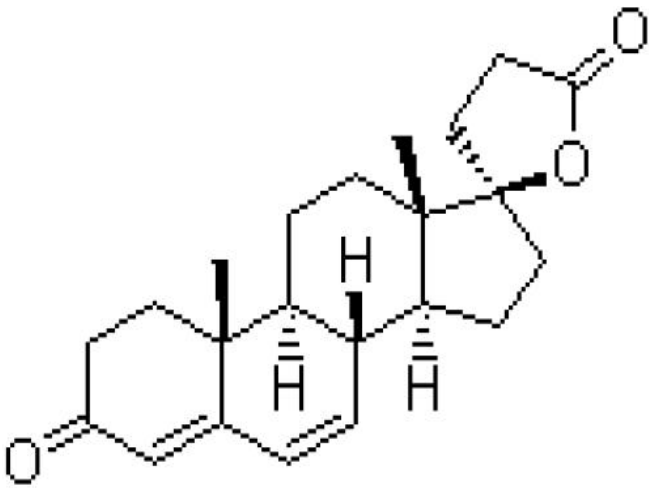


Spironolactone (SL)

7α-(acetylsulfanyl)-3',4'-dihydrospiro [androst-4-ene-17,2'(5'*H*)-furan]-3,5'-dione



Impurity 4 –Canrenone (imp 4)

17-hydroxy-3-oxo-17a-pregna-4,6-diene-21-carboxylic acid gamma-lactone

Figure 1 :Chemical structure of Torsemide, impurity 1, impurity 2, impurity 3, spironolatone and impurity 4

SL is simultaneously determined with triamterene, furosemide and hydrochlorothiazide by HPLC[19] SL is determined from a tablet formulation in combination with hydrochlorothiazide[20]. Many methods are available for determination of TD and metabolites by HPLC are using Cyano column[21], a cyclodextrin assisted capillary electrophoretic method[22], capillary electrophoresis with diode array detection[23], in human urine using electrochemical detection[24], in plasma[25,26], in plasma and urine by solid phase extraction[27] and in human plasma using monolithic column[28].

TD in formulation is determined by spectrometry[29]. Compendial methods by HPLC are available for both TD and SL individually[30]. However to the best of our knowledge no stability indicating LC method has been developed for simultaneous determination of related substances and assay of TD and SL from formulation.

EXPERIMENTAL

Chemicals and reagents

Working standard of TD (99.4 %) purity and SL (99.3 %) purity and impurity standards were obtained from Ipca laboratories, Mumbai, India. Marketed formulation Dylor plus 10 containing 10 mg TD and 50 mg SL were purchased from local market and used for the study. LC grade methanol, triethylamine and orthophosphoric acid were procured from Merck Mumbai, India. High purity deionized water was obtained from Millipore Milli Q plus water purification system (Milford, USA).

Instrumentation

The LC system used were PDA 2996, Waters and equipped with quaternary gradient pumps with auto sampler and auto injector (Alliance 2695, Waters, Milford, MA, USA) connected with a photo diode array detector (PDA 2996, Waters, Milford, MA, USA) controlled with Empower software (Waters).

Chromatographic Conditions

The separation was achieved on Hypersil BDS C8 column,(USP L7 column) (150 × 4.6 mm, 5 µm), using a mobile phase containing an equal quantities of 0.1 % Tri ethyl amine, pH adjusted to 5.0 using ortho phosphoric acid and methanol respectively. The mobile phase thus prepared was filtered through a 0.45 µm nylon membrane and degassed with sonication for 5 min. The mobile phase flow rate was 1.0 mL min^{-1} and wavelength 260 nm. The injection volume was 20 µL. Diluent used during the preparation of the standard and test sample was mixture of water: methanol (50: 50, *v/v*).

Preparation of Standard Solutions

A stock solution of SL (0.5 mg mL^{-1}) and TD (0.5 mg mL^{-1}) was prepared by dissolving an appropriate amount in diluent. Working solutions containing 50 µg mL^{-1} SL and 10 µg mL^{-1} TD were prepared from this stock solution for determination of assay. The above

stock solution was diluted suitably to obtain a concentration of 1 μg mL^{-1} each of TD, imp1, imp 2, imp 3 and 5 μg mL^{-1} each of SL and imp 4 and used as standard solution for related substances test.

Sample solutions

Ten whole tablets were weighed, transferred to a 500 mL volumetric flask, 10 mL of water was added to were disperse the tablets. 300ml of diluent was then added to the flask and was shaked for 30 min using a wrist action shaker for complete extraction of analytes. The solution thus prepared was sonicated for 30 min and diluted to volume to give a solution containing 1000 μg mL^{-1} of SL and 200 μg mL^{-1} of TD. This solution was filtered through a 0.45 μm pore size PVDF syringe filter and used for determination of related compounds. This solution was suitably diluted to obtain concentration of 50 μg mL^{-1} of SL and 10 μg mL^{-1} of TD and used for Assay of SL and TD.

RESULTS AND DISCUSSION

Method development and optimization

Literature survey showed pKa value of TD as 7.1 and SL as about 2.3. This made method development challenging primarily due to wide range of pKa value. Selecting a particular pH of mobile phase would make either of these drugs exist in ionized or non ionized forms. If ionized, the degree of ionization would greatly affect their chromatographic retention in RPLC. This is so, as typically these ionic form do not partition well between hydrophobic stationary phase such as C18 and mobile phase. Thus resulting into significantly lower k' (capacity factor). Low pH suppresses the ionization of most acidic analytes resulting in their higher retention. This was evident as acidic mobile phases such as that of 0.1 % glacial acetic acid in water: methanol in ratio 45: 55 (% *v / v*) respectively using Waters symmetry C18, 250 x 4.6 mm, 5 μ column, resulted in retention of SL and higher asymmetry with respect to peak due to TD. Peak modifiers such as triethylamine were then introduced in the mobile phase to reduce peak tailing caused by interaction of basic analyte TD with acidic surface silanols keeping the other chromatographic conditions same. To minimize retention of SL comparatively lower hydrophobic stationary phase such as C8 and length, 150 x 4.6 mm, 5 μ column was selected. This resulted in elution of TD in void volume; less than 2.0 min. Mobile phase composition 60: 40 (% *v / v*) was therefore tried, this gave good resolution of 8.6 between TD and SL. The method development involved two critical steps; first step being basic separation of TD and SL and second was specific separation with four studied impurities. For the second step in method development, when impurity mixture was injected in this set up, it did not show satisfactory resolution between imp 2 and TD. So methanol concentration was lowered to 50 % (v/v) but still the resolution between imp 1 and imp 3 was unsatisfactory. The increase in concentration of aqueous phase resulted in higher retention of SL. Mobile phase pH was altered to achieve the desired resolution. It was observed that at lower pH, resolution between critical pair imp2 and 3 is lower. As pH increased, the resolution also increased. 0.1 % triethyl amine adjusted to pH 5.0 with OPA and 50 % methanol was selected as optimum condition which resulted in good separation of all studied impurities and unknown degradants. The UV wavelength, 260 nm was selected for detection which is at the isosbestic point of SL and its imp 4 and also all the components found to have reasonable response to achieve the LOQ value below 0.1 % of test concentration.[31]. Typical chromatograms obtained with the developed method are as per (Figure 2) and system suitability parameters are listed in TABLE (1). Once the critical steps of method development was established, the chromatographic conditions were challenged for specificity. The specificity of a method is its suitability for analysis of a substance in the presence of potential impurities[32]. Stress testing of a drug substance can help identify likely degradation products, which can in turn, help establish degradation pathways and the intrinsic stability of the molecule. The specificity of the LC method for SL and TD was determined in the presence of four impurities and degradation products. Forced degradation of SL and TD was also performed to provide an indication of the stability-indicating properties and specificity of the method[33,34]. The tablet formulation was exposed to stress studies along with active pharmaceutical ingredients. The stress conditions chosen to achieve degradation included acid hydrolysis (0.1 M HCl/1M HCl), basic hydrolysis (0.1 M NaOH) and oxidation (10% H_2O_2) along with light (conducted

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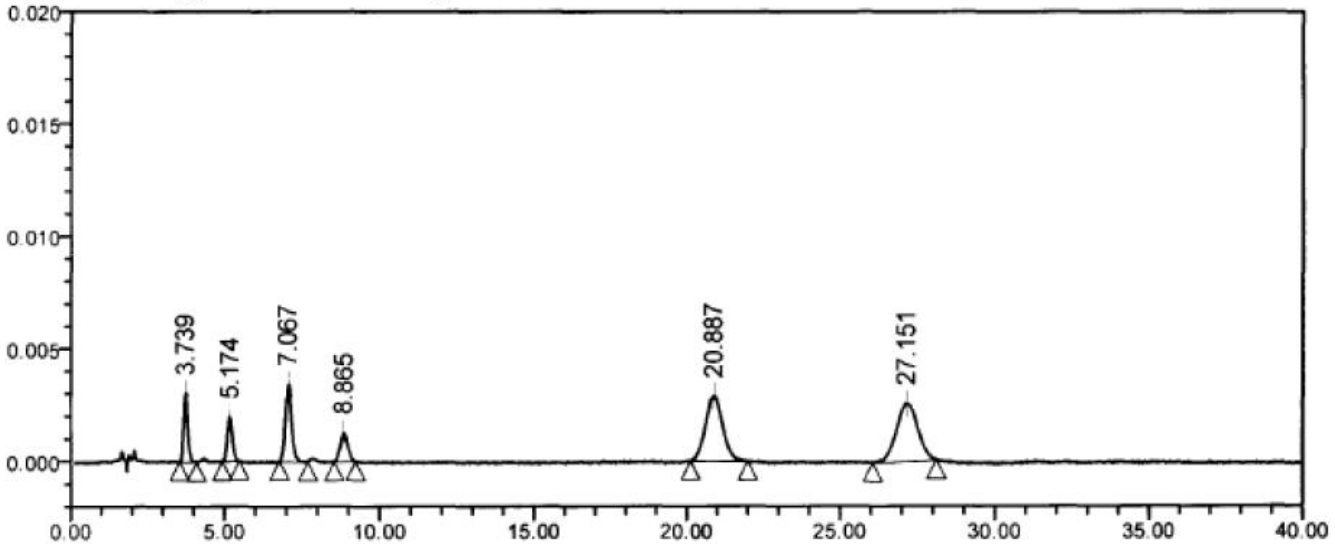
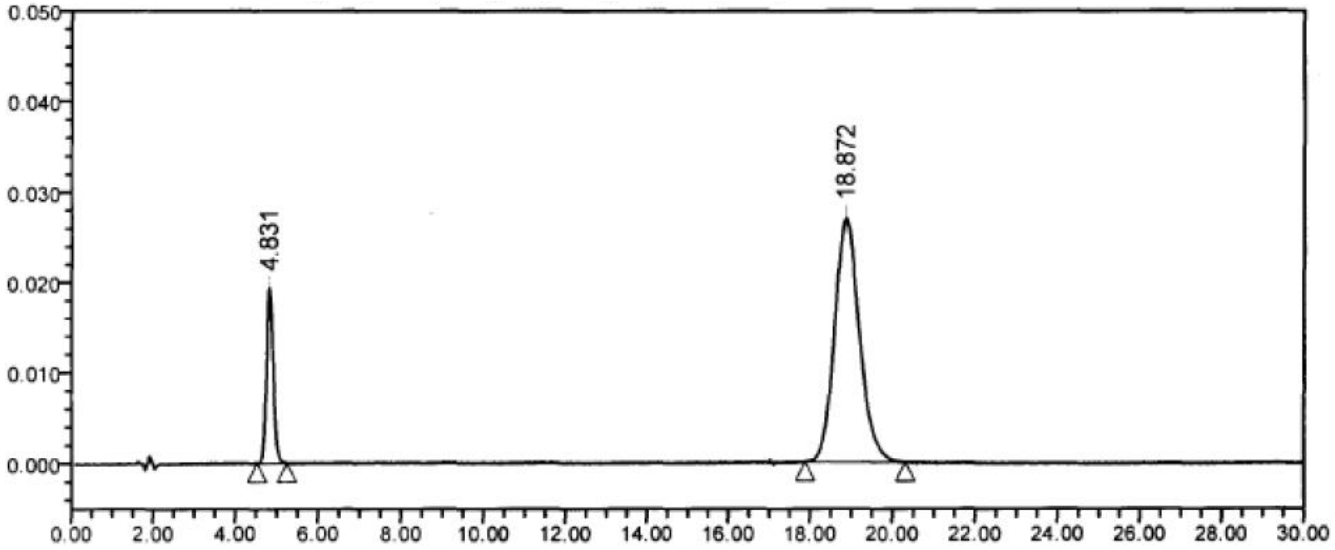
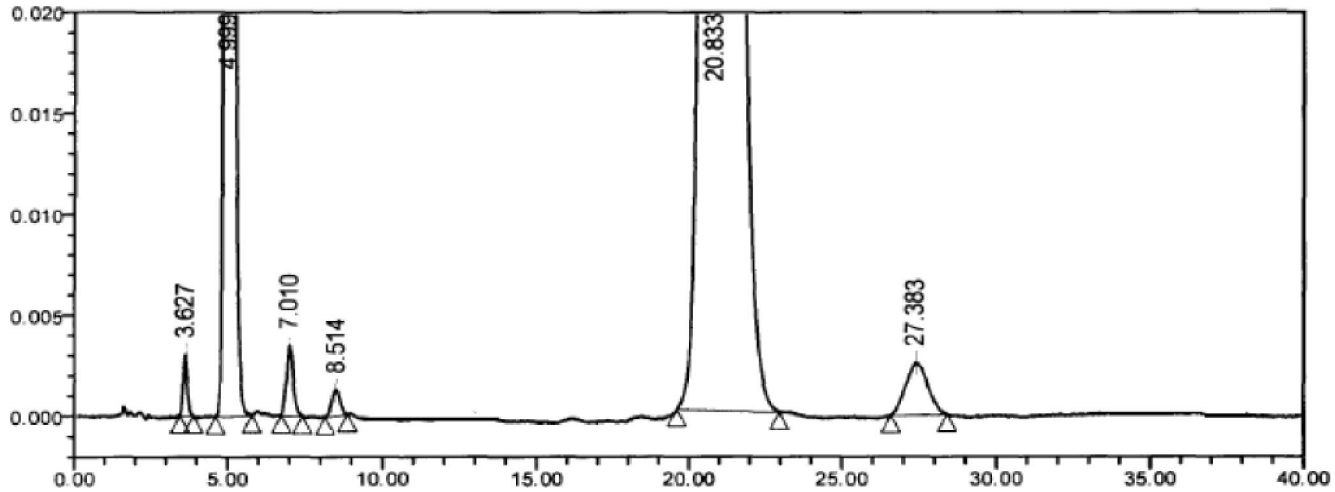


Typical chromatogram of Impurity mixture at 0.5% of test concentration, Order of Elution: Imp 1, TD, Imp 2, Imp 3, SL, Imp 4.



Typical chromatogram at Assay concentration



Typical chromatogram spiked with impurities at 0.5% level

Figure 2 : Typical chromatograms

TABLE 1 : Validation study Summary

Parameters	RelatedCompounds						Assay	
	Imp1	TD	Imp2	Imp3	SL	Imp4	TD	SL
Retentiontime(min)	3.7	5.1	6.9	8.7	20.5	26.6	4.8	19.5
Tailingfactor	1.2	1.0	1.2	1.0	1.1	1.0	1.2	1.2
Resolution(USP)	-	5.2	5.3	4.1	15.5	5.7	-	20.4
Theoreticalplates	3862	4126	5544	4888	6693	7081	4135	4679
Linearityrange(μgmL^{-1})	0.1-1.5	0.1-1.5	0.1-1.5	0.1-1.5	0.5-7.5	0.5-7.5	1to20	5to100
LOD(μgmL^{-1})	0.048	0.043	0.025	0.064	0.022	0.031	NA	NA
LOQ(μgmL^{-1})	0.14	0.13	0.08	0.18	0.65	0.94	NA	NA
Slope(b)	26868.14	23185.64	43434.4	17644.71	23623.78	24372.14	2634.4	24791.82
Intercept(a)	-440.74	-462.82	-1168.34	-116.74	-3277.7	-2768.26	-5025.28	-59100.6
Correlationcoefficient®	0.9990	0.9949	0.9977	0.9913	0.9984	0.9983	0.9999	0.9999
Residualsumofsquares(r^2)	0.9981	0.9899	0.9953	0.9846	0.9968	0.9966	0.9999	0.9999
Responsefactor	1.16	1.0	1.87	0.76	1.0	1.03	NA	NA
CorrectionFactor	0.86	1.0	0.53	1.31	1.0	0.97	NA	NA
MeanAccuracy(%)	100.1	NA	102.1	97.2	NA	102.1	99.8	100.0
Precision:Meanvalue(%)							99.3	99.9
Precision:RSDforn=6(%)	1.31	NA	3.81	1.71	NA	3.12	0.34	0.23

as stipulated in ICH Q1B) and heat at 60 °C. For studies of the effects of heat and light the study period was about 7 to 10 days whereas for acidic, basic, and aqueous hydrolysis and oxidation it was about 2 h. The purity angle is less than the purity threshold limit obtained in all stressed samples by using Waters Empower software ensured analyte peak homogeneity as required by ICH Q2R(2).The peak purity obtained from stressed samples using PDA detector confirmed method to be stability indicating. The assay of stressed samples was calculated against reference standard using external standard method and the mass balance (% assay + % impurities + % degradation products) was demonstrated. When TD and SL were exposed to heat, degradation was not observed. Similarly exposure to photolytic degradation, TD did not show any degradation but SL showed degradation with change in physical appearance from white to yellow. Also Drug product exposed to photolytic studies showed change in color from white to yellow. TD was found to degrade in acidic hydrolysis as well as in oxidation forming imp 2 at about 8 to 15% level. The presence of imp 2 as a degradant was confirmed by spiking imp 2 in degraded samples. Stress studies on SL in basic hydrolysis, acidic hydrolysis and oxidation concluded that imp 4 to be one of the major degradants during basic hydrolysis. (Figure 3). The mass balance for the stressed samples was close to 99.6 for TD and 99.5 for SL TABLE (2).

Method validation

Method validation was carried out as per ICH guidelines for parameters such as Precision, linearity, accuracy, Limit of detection and quantitation, robustness, response factor and stability in solution.

Precision

The precision of the related substance method was checked by six fold analysis of tablet sample spiked with 0.5% of each of the four impurities. The RSD (%)

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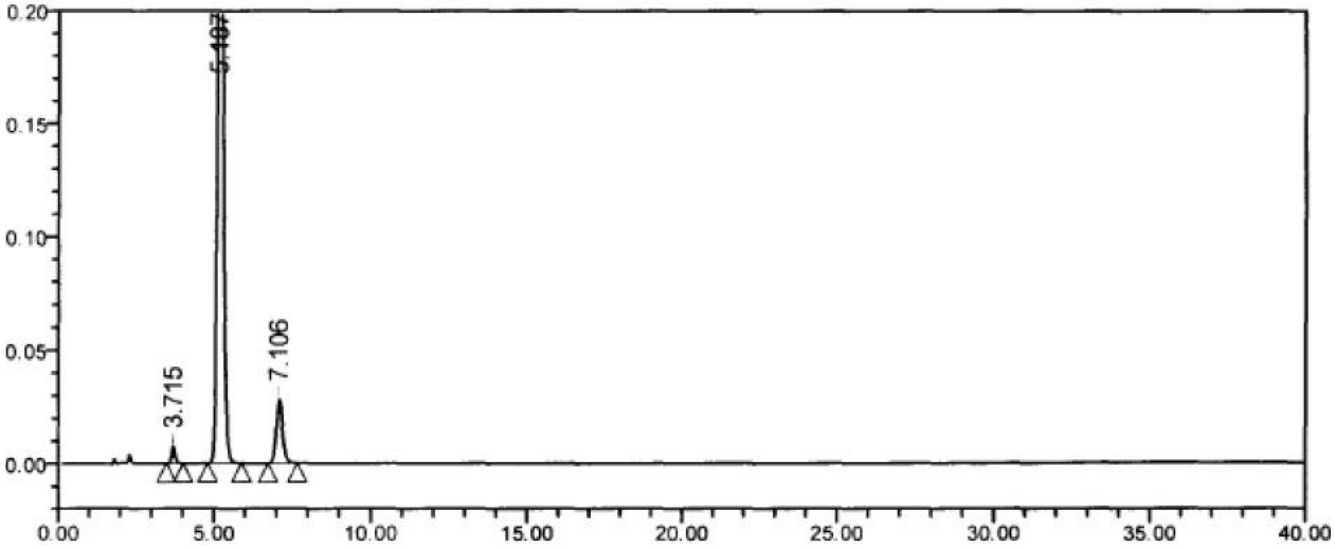
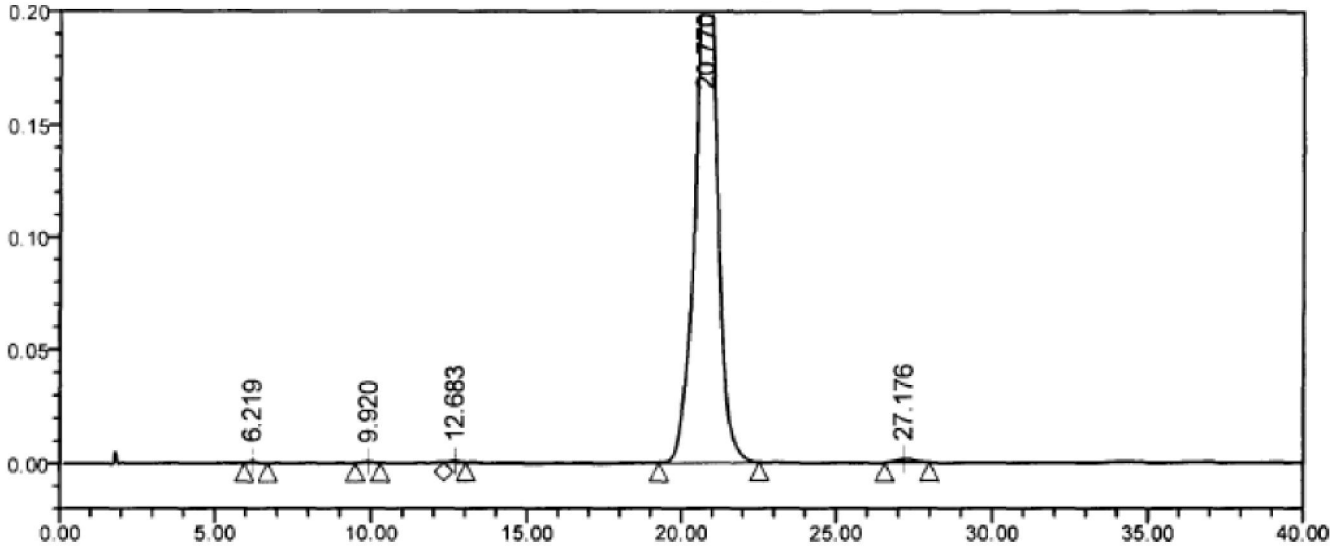
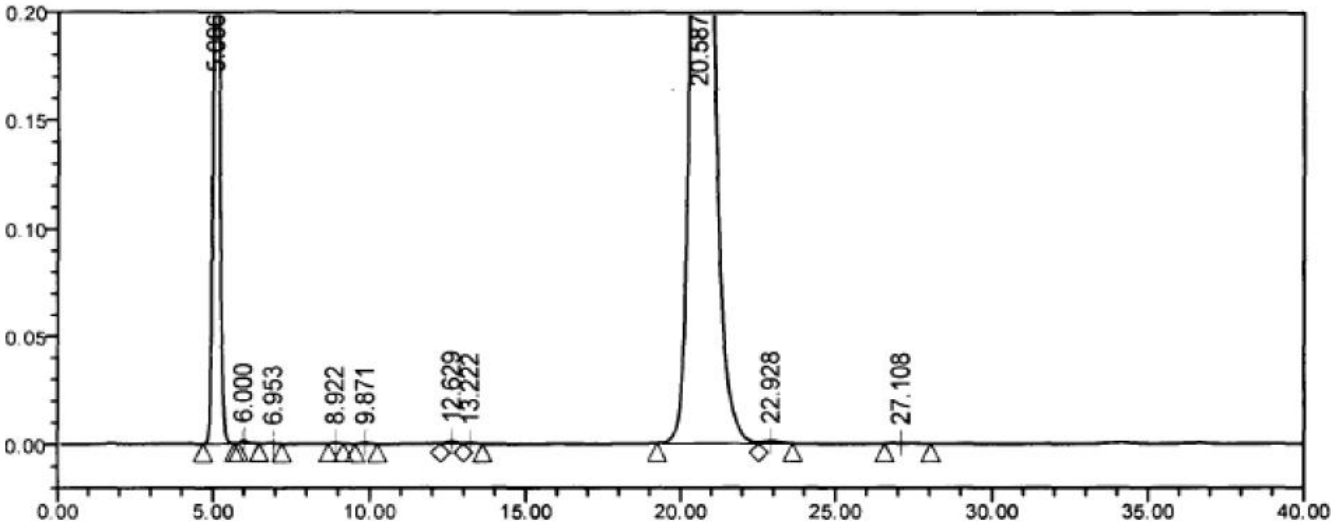


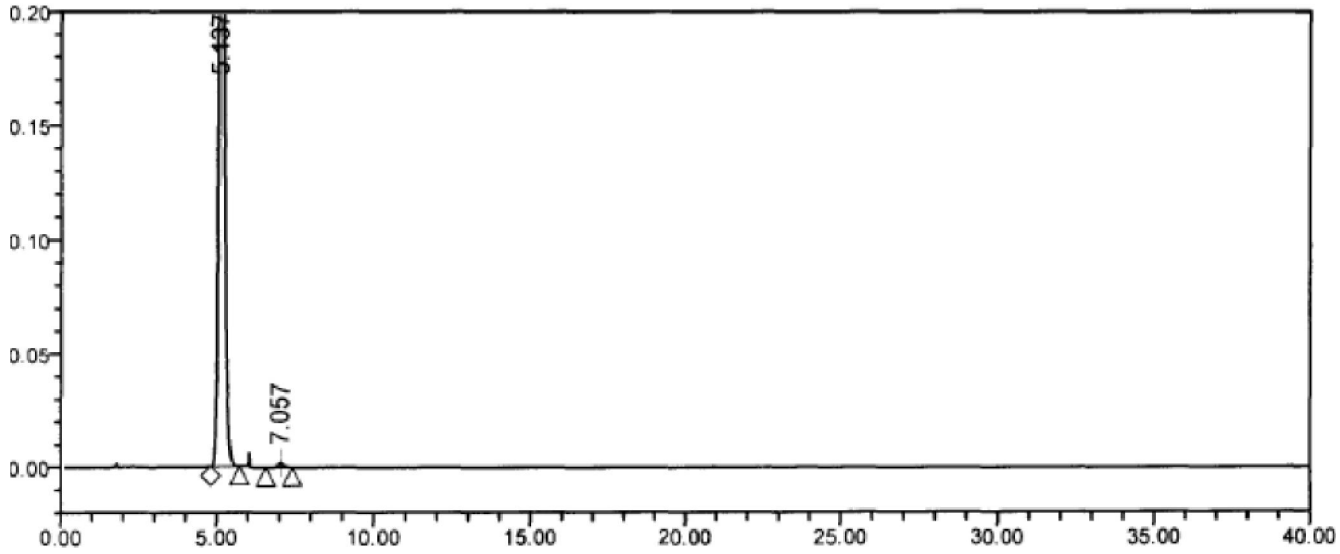
Acidic hydrolysis of Torsemide



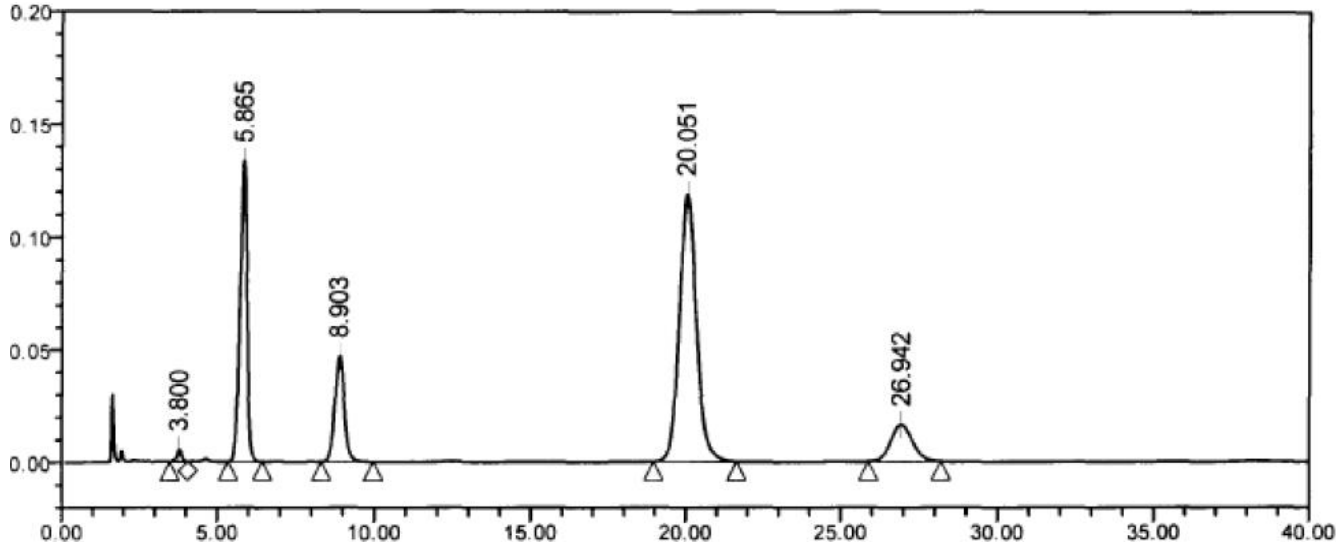
Acidic hydrolysis of Spironolactone



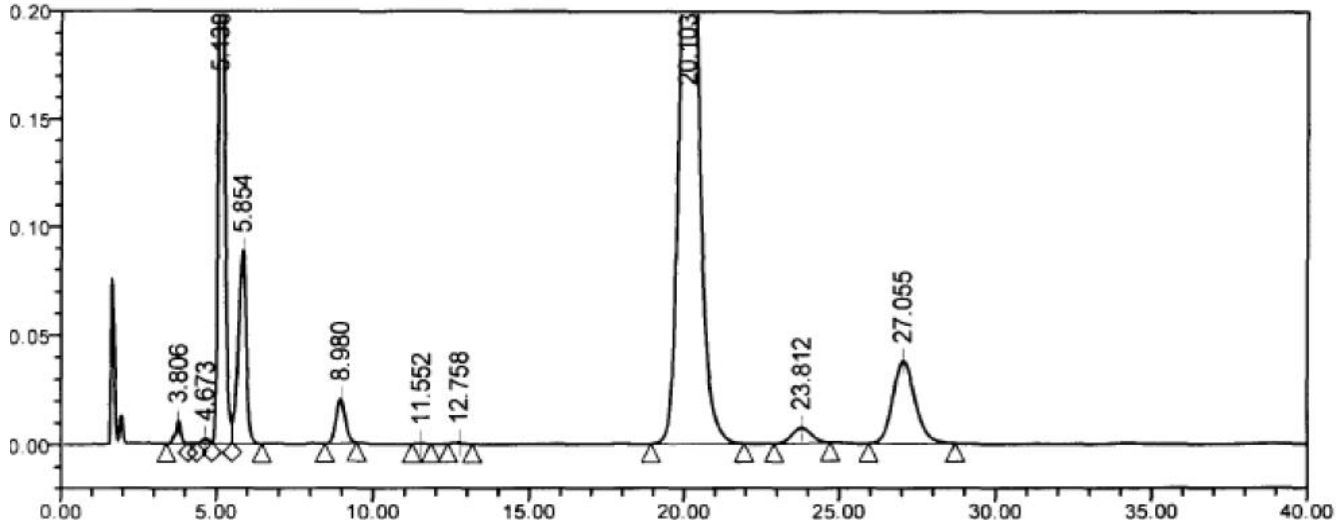
Acidic hydrolysis of Tablet formulation



Basic hydrolysis of Torsemide



Basic hydrolysis of Spironolactone



Basic hydrolysis of Tablet formulation

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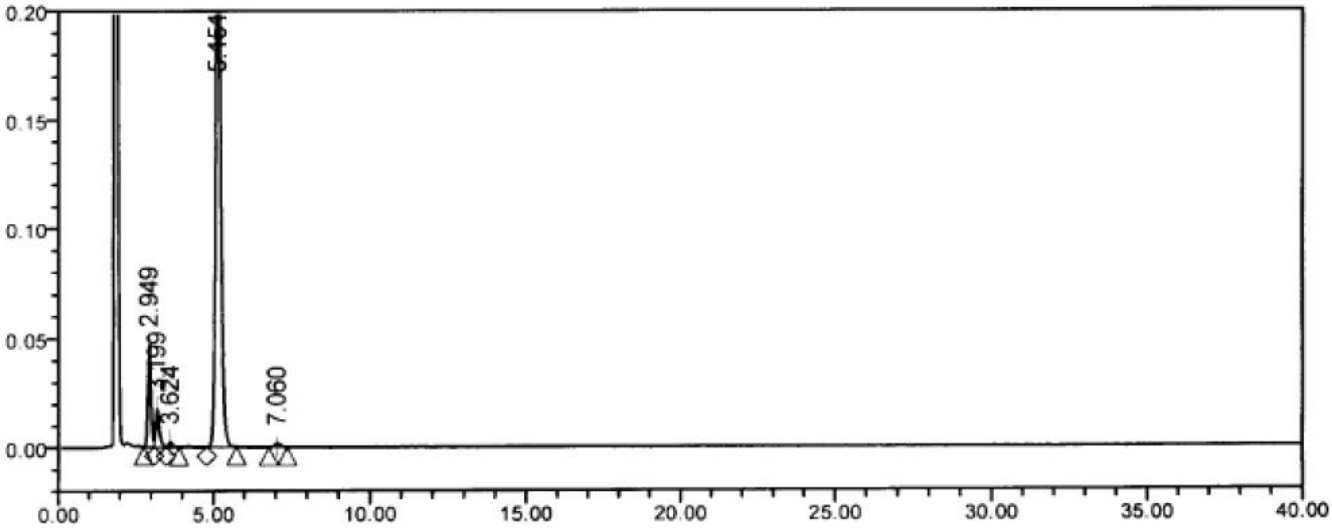
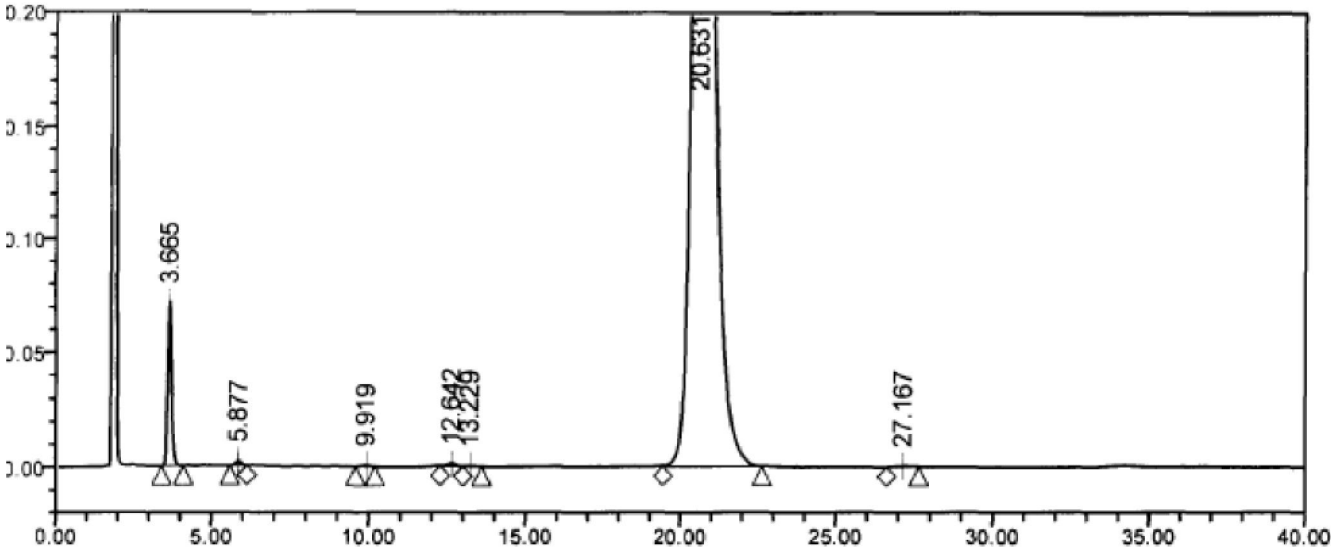
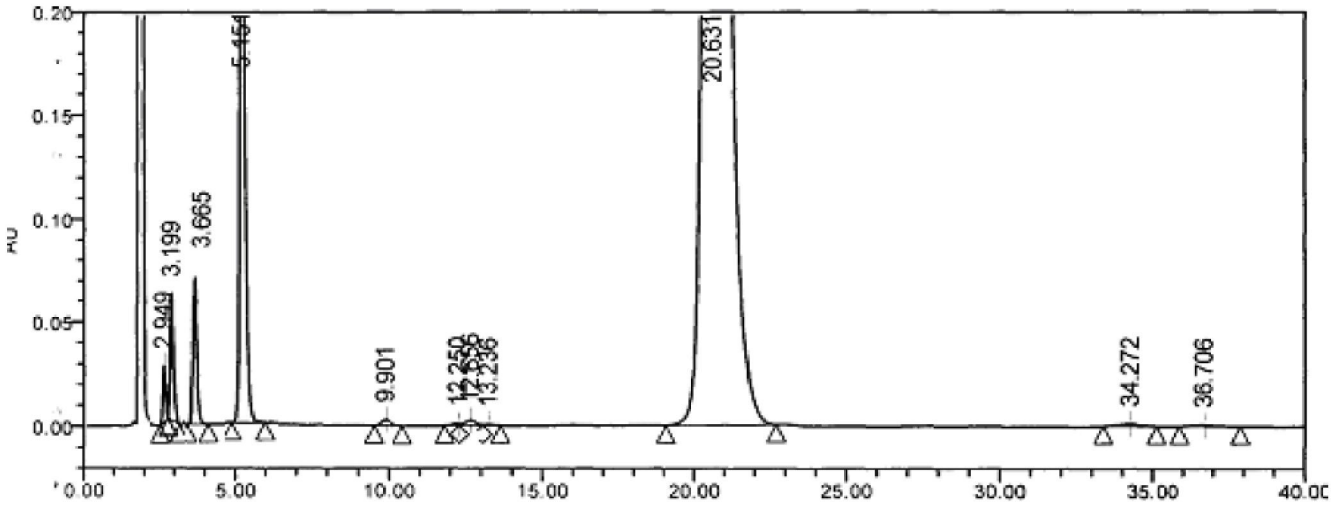


Oxidation of Torsemide



Oxidation of Spironolactone



Oxidation of Tablet formulation

Figure 3 : Chromatograms from forced degradation study

TABLE 2 : Summary of results of forced degradation experiments

Stress condition	Time	Assay of Active	Mass balance (% assay + % degradation product)	Remarks (RRT wrt TD)	Peak purity
Torsemide					
Acidic hydrolysis (1N HCL)	2 h	91	100	Imp 2 formed as a degradant.	Pure
Basic hydrolysis (0.1N NaOH)	2 h	98	98.8	No major degradation found.	Pure
Oxidation(10% H_2O_2)	2 h	84.1	99.7	Unknown imp at RRT 0.6 formed as a major degradant and imp 2 formed.	Pure
Thermal treatment at 60^0 C	7 days	99.2	99.7	No degradation found.	Pure
Light (photolytic degradation) ICH Q1B	10 days (1.2 million lux h)	99.5	99.7	No degradation found.	Pure
Spironolactone					
Acidic hydrolysis (0.1N HCl)	30 min	98.1	99.8	No major degradation found.	Pure
Basic hydrolysis (0.1N NaOH)	30 min	66.8	100.2	Unknown imp at RRT1.13 as a major degradant formed was well separated from TD. Unknown imp at RRT 1.75 formed was closely eluting with imp 3 but well separated. Imp 4 was also formed.	Pure
Oxidation(10% H_2O_2)	30 min	97.1	98.6	Unknown imp at RRT 0.71 formed was closely eluting with imp 1 but well separated.	Pure
Thermal treatment at 60^0 C	7 days	98.8	100	No major degradation found.	Pure
Light (photolytic degradation) ICH Q1B	10 days (1.2 million lux h)	96.5	98.7	No major degradation found The physical appearance of the API changed from white to yellow.	Pure

of peak area for each impurity which was within 5% confirmed precision of the method. The precision of the assay was evaluated by performing six independent assays of a test sample and quantifying using external standard method using reference standard. The RSD (%) of the six results were 0.34 and 0.23 respectively for TD and SL, confirmed method to be precise for assaying of TD and SL in presence of its impurities.

To evaluate the intermediate precision (ruggedness) of the method, the analysis was performed on a different day using a different instrument in the same laboratory. RSD (%) of TD and SL in the study of intermediate precision was 0.37 and 0.42 respectively confirmed method repeatability.

Limit of detection (LOD) and quantification (LOQ)

LOD and LOQ for the four impurities and analytes were estimated as the amounts for which the signal-to-noise ratios were 3:1 and 10:1 respectively. The study was performed by injecting a series of dilute solutions of known concentration within the developed chromatographic conditions[35]. The limit of detection for all studied impurities was below 0.03 % and limit of quantitation was below 0.1 % of the test concentration. TABLE (1).

Linearity

Solution for testing linearity for the related substances were prepared by diluting the impurity stock solution to seven different concentrations from the LOQ to 150 % of the permitted maximum level of the impurity (i.e. the LOQ and 0.10, 0.25, 0.375, 0.5, 0.6 and 0.75 % for an analyte concentration of 1000 μg mL^{-1} SL and 200 μg mL^{-1} for TD). The correlation coeffi-

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cient, slope, and *y*-intercept for each impurity and analytes assured linearity in the studied range. The linearity of the assay method was determined at six concentrations from 10 to 200% of the analyte concentration (1 to 20, μg $mL^{-1}$for TD and 5 to 100 μg mL^{-1} for SL) were prepared from the stock solution. Least-squares linear regression analysis was performed on peak area and concentration data. TABLE (1).

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentrations, 40, 50, and 60 μg mL^{-1} for SL and 8, 10, and 12 μg mL^{-1} for TD respectively. % recovery within 98.0 to 102.0% confirmed accuracy of the method. For the impurities, recovery was determined in triplicate for 0.4, 0.5, and 0.6 % of the analyte concentration (1000 μg mL^{-1} SL and 200 μg mL^{-1} for TD) and % recovery of the impurities was calculated found within 85 to 115%. TABLE (1).

Robustness

To determine the robustness of the method the experimental conditions were deliberately altered and checked for the system suitability criteria ie resolution between closely related peaks and retention of SL and imp 4. The chromatographic conditions which were altered are flow rate (±0.2 mL $min^{-1)}$; of mobile phase(±0.2 units); methanol concentration in mobile phase (±10 % absolute). During method development extreme pH (pH3.0) and methanol concentration (40 %) studied helped to define the design space within which method is robust. TABLE 3.

Relative response factor (RRF) and correction factor (CF) study

Response factor is a relative term, being the response of equal weights of one substance relative to that of another in the conditions described in the test. The RRF value for given impurity as the ratio of response of peak at a particular concentration to that of analyte peak at that concentration. The RRF values less than 0.2 and more than 5.0 are not acceptable as per European pharmacopoeia. In such cases there is a need for change in chromatographic parameters like wavelength or different method of visualization is used[36]. The extrapolation of linearity study was done to determine RRF and CF[37]. TABLE 1.

Stability in solution

The stability of SL and TD and their impurities in solution was determined by keeping test solutions of the sample and reference standard and spiked sample solution volumetric flasks at room temperature for 72 h at bench top and withdrawn at regular time intervals to assay the analytes. The absolute difference of assay values within 2% and impurities from initial value con-

TABLE 3 : Results from Robustness study

Condition	Variation	Resolution			Retention time		Remarks
		Imp1-TD	TD-Imp2	Imp2-Imp 3	SL	Imp4	
Mobile phase pH (±0.2 units)	5.2	4.30	4.47	3.67	17.4	22.2	Method is robust for small variations At lower pH, elution of impurities of torsemide gets reversed and resolution is lost.
	5.0	4.57	4.66	3.98	18.92	24.11	
	4.8	4.43	4.51	3.51	17.61	22.48	
	3.0	Elution pattern is reversed- Imp 2 eluted before TD			19.03	24.2	
Mobile phase composition (±5% methanol)	55	3.4	4.6	1.8	9.67	12.42	Methanol concentration is very critical for elution of SL and Imp 4.
	50	4.57	4.66	3.98	18.92	24.11	
	45	5.36	4.68	4.72	37.77	47.55	
	40	6.60	4.78	6.49	86.92	106.15	
Flow rate(±0.2 mL min^{-1})	1.2	4.50	4.52	3.50	15.12	19.29	Flow rate did not affect critical resolution and elution.
	1.0	4.57	4.66	3.98	18.92	24.11	
	0.8	4.50	4.54	3.61	22.7	28.93	

TABLE 4 : Results (%) from stability study on Marketed sample: Dytor plus 10

Initial	Related substances by HPLC						Assay	SL
	Imp 1	Imp2	Imp3	Imp4	Unk max	Total imp	TD	
	BLQ	**0.014**	**0.04**	**0.06**	**0.142**	**0.590**	**98.8**	**100.4**
storage condition 40 ± 2^{0}C and RH 75 ± 5%								
1st month	BLQ	0.06	0.04	0.08	0.144	0.890	98.2	99.1
2nd month	BLQ	0.08	0.039	0.10	0.151	0.901	98.1	99.8
3rd month	BLQ	0.12	0.039	0.129	0.158	0.920	98.0	99.9
storage condition 30 ± 2^{0}C and RH 65 ± 5%								
1st month	BLQ	0.02	0.040	0.08	0.147	0.840	98.9	99.8
2nd month	BLQ	0.04	0.04	0.08	0.145	0.860	98.2	98.2
3rd month	BLQ	0.05	0.038	0.108	0.149	0.860	98.8	99.8
storage condition 25 ± 2^{0}C and RH 60 ± 5%								
1st month	BLQ	0.020	0.040	0.070	0.150	0.770	98.5	99.1
2nd month	BLQ	0.03	0.039	0.081	0.145	0.780	98.1	99.2
3rd month	BLQ	0.04	0.035	0.098	0.144	0.790	98.5	99.5

cluded solution stability for 72 h for assay however related substances was found stable upto 24h.

Application of the method to real time stability studies as per ICH guidelines confirms the stability indicating power of the method. TABLE (4).

CONCLUSION

The isocratic RP-LC method for simultaneous determination of TD, SL and related substances in combination drug product is precise, accurate and specific. The results obtained from the method were satisfactory. The method is stability indicating and can be used for routine analysis of production samples and stability studies.

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