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Research Article

VALIDATED STABILITY-INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE ESTIMATION OF TORSEMIDE

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ABSTRACT

Objective: This assessment depicts the strength of exhibiting reverse-phase high performance liquid chromatography (RP-HPLC) method for the estimation of torsemide in pharmaceutical estimation structures.

Methods: In the present work, total protein-HPLC technique has been produced for the estimation of torsemide active pharmaceutical ingredient (API). Constrained degradation HPLC strategy was created with versatile mobile phase of methanol:water in the proportion of 90:10 v/v. The stream pace of 1 ml/min was utilized on Inertsil ODS 3V segment (250 mm×4.6 mm, 5 µm molecule size).

Results: The retention time of torsemide was seen at 8.267 min, method was validated for all validation parameters as per the International Council for Harmonization guidelines. The linearity range was 10–60 µg/ml, correlation coefficient was 0.9993, and percentage relative standard deviation in the precision studies was <2%, with percentage recovery 100.56–101.03 (within acceptable range of 98–102%). The assay result was found to be 100.88% (i.e., within 95–105%), passes the specifications for robustness parameters. Limit of detection of torsemide was found to be 0.0162 µg/ml and limit of quantitation of torsemide was found to be 0.0534 µg/ml.

Conclusion: The medication was exceptionally delicate to antacid pursued by at risk to corrosive, photolytic, warm, and oxidative conditions. The created and approved method showing HPLC technique is observed to be direct, exact, precise, explicit, and powerful. Henceforth, the technique can be utilized routinely for the estimation of torsemide API.

Keywords: Torsemide, Reverse-phase high-performance liquid chromatography, Forced degradation.

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INTRODUCTION

1-Isopropil-3-[4-(3-methylphenylamino)-3-pyridinesulphonyl] urea (torsemide) is the most dynamic agent of the novel anilinopyridine sulfonylurea subsidiaries class diuretics. It has been found to have impacts on water and electrolyte discharges. As a result of its site of activity obstructing the sodium and chloride reabsorption at the circle of Henle, it tends to be arranged by definition as a circle diuretic. Likewise, its diuretic profile intently takes after that of circle diuretics. Be that as it may, as opposed to the common circle diuretics of the furosemide type, torsemide demonstrates a few points of interest by its generously longer organic half-life, longer term of activity, and significantly less articulated kaliuretic and phosphaturic impacts. Torsemide is appropriate for a wide range of various signs that are viable for the treatment of hypertension in the low portion of 2.5 mg and up to the treatment of high-grade renal disappointment with the high portion of 200–400 mg [1-5].

Among several strategies utilized for the estimation of torsemide high-performance liquid chromatography (HPLC) turn around stage superior generally used to approve International Council for Harmonization (ICH) rules. The motivation behind this examination was to build up a straightforward, delicate, specific, and reproducible explanatory strategy for the quantitative estimation of medication. This report portrays a particular and exact HPLC strategy for the evaluation of torsemide [6-8].

METHODS

Preparation of standard drug solutions

Accurately 10 mg of the torsemide pure drug was weighed and transferred into 10 ml clean dry volumetric flask. The volume was made up to the mark with methanol (1000 µg/ml). One milliliter of above solution was transferred to a 10 ml volumetric flask. The volume was made up to the mark with mobile phase (100 µg/ml). Two milliliters of above solution were transferred to a 10 ml volumetric flask. The volume was made up to the mark with mobile phase to get 20 µg/ ml concentration of torsemide. The solutions were injected under the above chromatographic conditions and peak areas were measured.

Chromatographic conditions

The mobile phase consisted of methanol:water in the ratio of 90:10; contents of the mobile phase were filtered before use through a 0.45 µ membrane filter and degassed for 15 min. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.0 ml/min and the injection volume was 20 µl. The eluents were monitored at 287 nm. The optimized conditions are shown in Tables 1 and 2 and Fig. 1.

Calibration of standards

Different volumes of stock solutions were accurately transferred into 10 ml volumetric flasks and diluted to mark to yield concentration range of 10–60 µg/ml for torsemide. Six solutions were prepared and the final volume was made up to the mark with mobile phase. The calibration curve was obtained by plotting the peak area against the concentration of drug shown in Table 3.

Method validation of torsemide [9-17]

Method of validation was performed in terms of specificity and selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, and robustness.

Table 1: Optimization of mobile phase for the determination of torsemide

S. No.	Composition of mobile phase (methanol:water) 1 ml/min flow rate	Retention time (R_t) in (min)	Asymmetry	Theoretical plates
1.	75:25	14.073	0.88	5014
2.	80:20	11.560	0.88	4435
3.	85:15	9.250	0.90	3917
4.	90:10	8.267	1.07	4706

Table 2: Optimized chromatographic conditions of torsemide

S. No.	Parameter	Optimized condition
1.	Mobile phase composition	Methanol and water in the ratio of 90:10
2.	Stationary phase	Inertsil ODS-3V (250 mm×4.6 mm i.d.,5 µ)
3.	Flow rate	1 ml/min
4.	Run time	15 min
5.	Column temperature	Ambient
6.	Volume of injection	20 µl
7.	Detection wavelength	287 nm
8.	Retention time of the drug	8.267 min

Table 3: Calibration of torsemide

S. No.	Concentration (µg/ml)	Peak area Mean±SD (n=3)	% RSD
1.	10	5,795,640±55,744	0.96
2.	20	11,349,652±65,566	0.58
3.	30	16,337,929±98,252	0.60
4.	40	22,532,479±105,323	0.47
5.	50	28,347,816±130,576	0.46
6.	60	33,623,461±66,358	0.20

RSD: Relative standard deviation, SD: Standard deviation

Table 4: Specificity and selectivity

Injection	R_t (min) of analytes	R_t (min) of degradants	Remarks
Blank	---	---	No component found in blank
Control	8.267	---	Method was specific
Degraded sample	8.787	1.910	Impurity was separated from the active pharmaceutical ingredient

Specificity and selectivity

Specificity is the ability of a method to discriminate between the intended analyte(s) and other components in the sample. Selectivity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients. The results are shown in Table 4 and Fig 2.

Linearity

The linearity of calibration curves (peak area vs. concentration) in pure solution was checked over the concentration ranges of about 10–60 µg/ml for torsemide. The eluting time was <15 min. The regression lines relating standard concentrations of drug using regression analysis, the calibration curves were linear in the studied range and equations of the regression analysis were obtained:

Y=560,937.5629x+31,681.4667, r^2=0.9993 for torsemide.

The mean±standard deviation (SD) for the slope, intercept, and correlation coefficient of standard curves (n=3) were calculated. The represented data are shown in Table 5 and Fig. 3.

Table 5: Linearity report of torsemide

S. No.	Parameter	Values for torsemide
1.	Linearity range	10–60 µg/mL
2.	Regression equation	Y=560,937.5629x+31,681.4667
3.	Correlation coefficient	r^2=0.9993
4.	Intercept	31,681.4667
5.	Slope	560,937.5629

Table 6: System precision of torsemide

Injection No.	Concentration (µg/ml)	Peak area at 287 nm	R_t (min)
1.	20	11,349,652	8.717
2.	20	11,256,927	8.565
3.	20	11,232,479	8.703
4.	20	11,306,123	8.761
5.	20	11,372,602	8.687
6.	20	11,320,925	8.724
Mean±SD		11,306,451±53,621	8.692±0.067
% RSD		0.47	0.77

RSD: Relative standard deviation, SD: Standard deviation

Precision

i. Repeatability: The precision of the method was evaluated by calculating the relative SD (%RSD) of peak areas of six replicate injections of standard concentrations. The average RSD of torsemide was found to be 0.47%. The results are shown in Table 6.
ii. Reproducibility: The RSD of peak areas of eight replicate injections for three different standard concentrations was calculated. The average RSD of torsemide was found to be 0.47%. The results are shown in Table 7.
iii. Intermediate precision (inter- and intraday): The precision expressed within the same laboratory on different days variability of analytical results. The RSD of peak areas of three replicate injections for three different standard concentrations was calculated. For intraday studies, the average RSD of torsemide was found to be 0.46%, and for interday studies, the average RSD of torsemide was found to be 0.25%. The results are shown in Table 8.

Accuracy

Accuracy of the method was determined by recovery experiments. To the formulation, the reference standards of the drug were added at the level of 80%, 100%, and 120%. The recovery studies were carried out 3 times and the percentage recovery and percentage RSD of the recovery were calculated and results are shown in Table 9.

$$\% \text{ Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

Assay of torsemide

Accurately 10 mg of torsemide pure drug was weighed and transferred into 10 ml volumetric flask. The volume was made up to the mark with methanol (1000 µg/ml). One milliliter of above solution was transferred into a 10 ml volumetric flask and volume was made up to the mark with mobile phase (100 µg/ml). From the above solution, 2 ml was taken into a 10 ml volumetric flask and volume was made

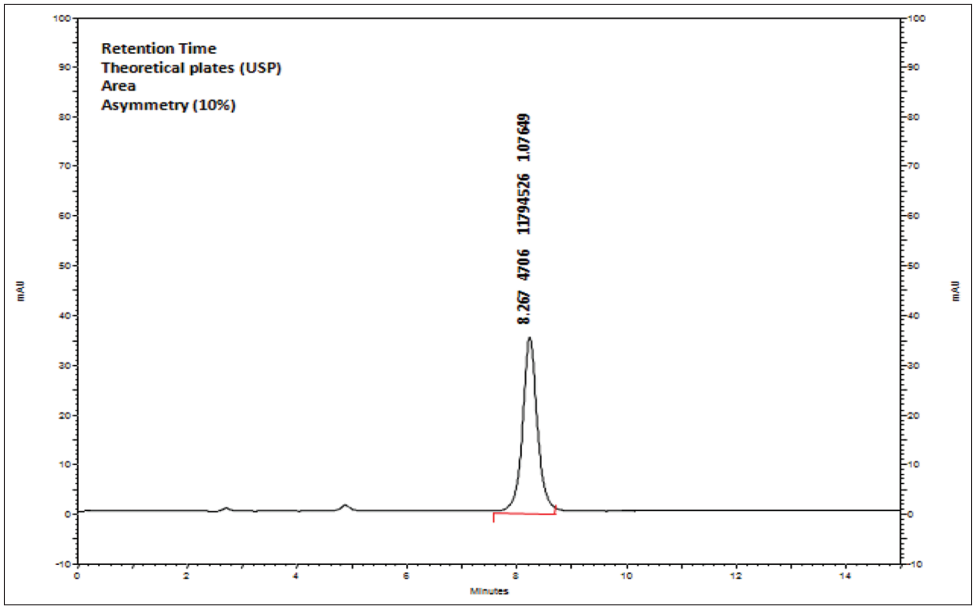

Fig. 1: Optimized chromatogram of torsemide

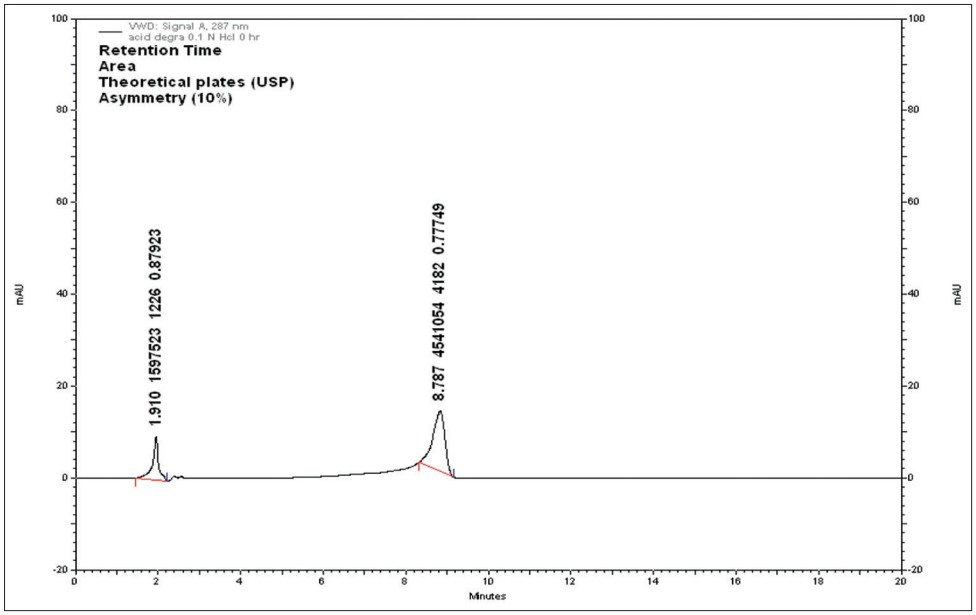

Fig. 2: Chromatogram of degraded sample – selectivity

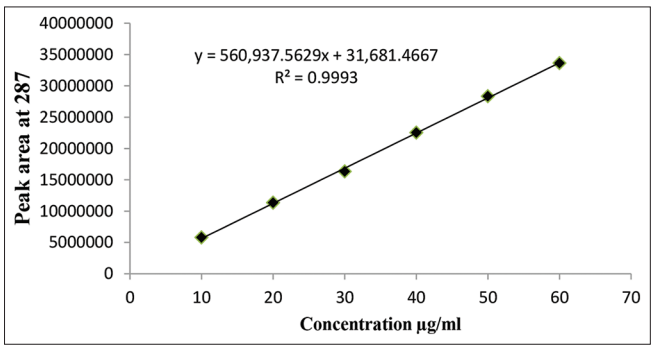

Fig. 3: Calibration curve of torsemide

up to the mark with mobile phase to get 20 µg/ml concentration of torsemide. The solution was injected under above chromatographic conditions and peak area was measured. The assay procedure was made triplicate and weight of drug taken for assay was calculated. The percentage of drug found, mean and SD were calculated, as shown in Table 10 and Fig. 4.

LOD and LOQ

The LOD and LOQ were performed on samples containing very low concentrations of analytes under the ICH guidelines. By applying the mathematical formula method, LOD was expressed by establishing the minimum level at which the analyte can be reliably detected. LOQ was considered as the lowest concentration of analyte in standards that can be reproducibly measured with acceptable precision. The LOD and LOQ values for torsemide are shown in Table 11.

Robustness

The optimum HPLC conditions set for this method have been slightly modified for samples of torsemide dissolved in the drug matrix as a means to evaluate the method robustness. The small changes include the flow rate (± 0.1ml/min), change in the composition of mobile phase

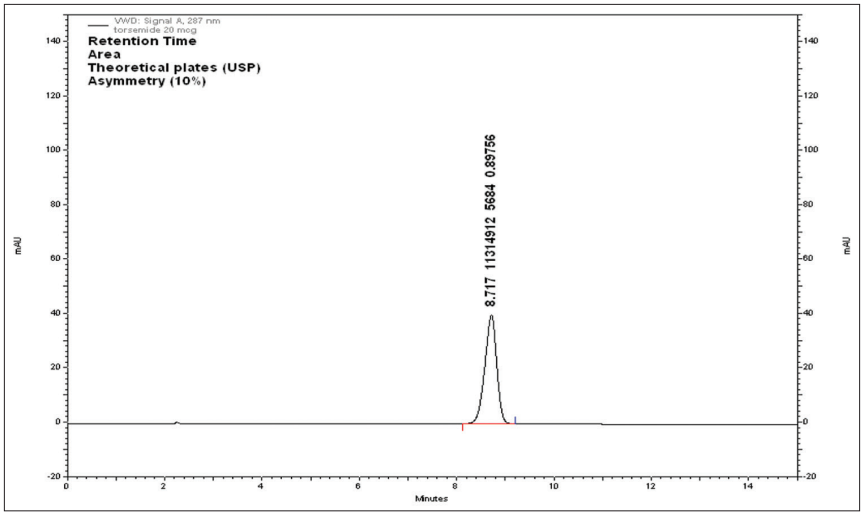

Fig. 4: Assay chromatogram of torsemide

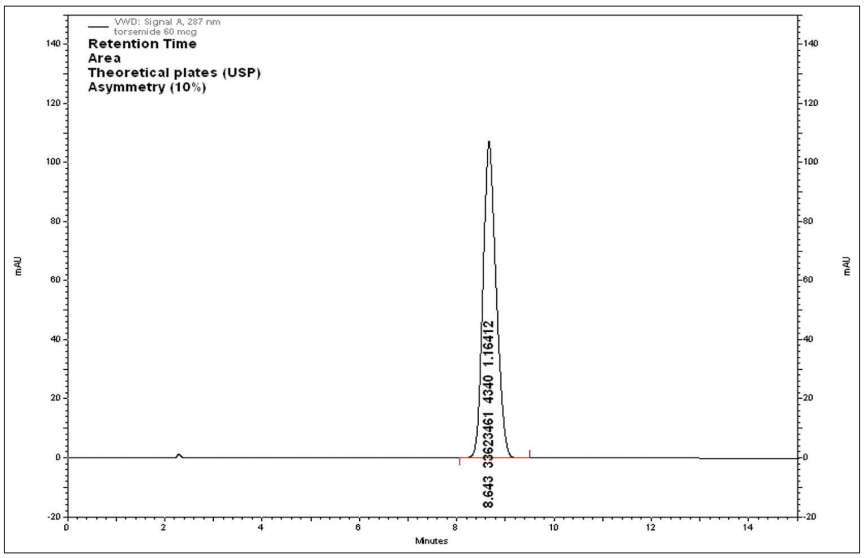

Fig. 5: Retention time of torsemide

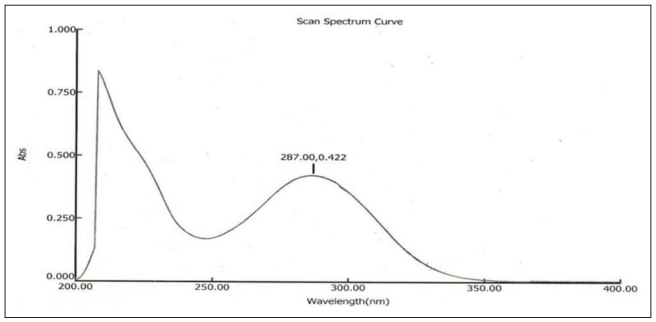

Fig. 6: Ultraviolet spectrum of torsemide

(± 2 ml), pH of the mobile phase (± 0.1 units), and detection wavelength (± 2 nm). The represented data are shown in Table 12.

System suitability

It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. The system suitability was assessed by five replicate analyses of the drugs at concentrations of 20 µg/ml of torsemide. Parameters such as plate number (n), tailing factor, and peak asymmetry of samples were measured, as shown in Table 13 and Fig. 5.

Table 7: Method precision of torsemide

Injection No.	Conc. (µg/ml)	Peak area at 287 nm	R_t (min)
1.	20	11,425,694	8.913
2.	20	11,390,623	8.786
3.	20	11,332,479	8.817
4.	20	11,402,581	8.823
5.	20	11,459,206	8.758
6.	20	11,290,285	8.905
7.	20	11,376,923	8.792
8.	20	11,418,267	8.826
Mean±SD		11,387,007±53,947	8.827±0.055
% RSD		0.47	0.62

RSD: Relative standard deviation, SD: Standard deviation

Forced degradation studies of torsemide

Degradation studies were carried out as per the ICH guidelines. The objective of this study was to find out the degradation products,

Table 8: Intra- and interday precision of torsemide

S. No.	Conc. (µg/ml)	Intraday precision		Interday precision	
		Peak area Mean±SD (n=3)	% RSD	Peak area Mean±SD(n=3)	% RSD
1.	10	5,874,475±41,107	0.70	5,933,123±19,579	0.33
2.	30	17,426,467±74,015	0.42	17,321,908±54,168	0.31
3.	60	33,717,306±94,642	0.28	33,737,146±39,434	0.12

RSD: Relative standard deviation, SD: Standard deviation

Table 9: Recovery studies of torsemide

S. No.	Pre-analyzed sample concentration (µg/ml)	Recovery level (%)	Amount added (µg/ml)	Amount of drug found (µg/ml), (n=3) mean±SD	% recovery	% RSD
1.	20	80	16	16.165±0.109	101.03	0.67
		100	20	20.112±0.146	100.56	0.73
		120	24	24.243±0.111	101.01	0.46

RSD: Relative standard deviation, SD: Standard deviation

Table 10: Assay of torsemide

S. No.	Active pharmaceutical ingredient (active pharmaceutical ingredient)	Concentration (µg/ml)	Amount found (µg), (n=3) Mean±SD	% assay	% RSD
1.	Torsemide	20 µg	20.176±0.084	100.88	0.42

RSD: Relative standard deviation, SD: Standard deviation

Table 11: LOD and LOQ of torsemide

S. No.	Drug	LOD	LOQ
1.	Torsemide	0.0162 µg/ml	0.0534 µg/ml

LOD: Limit of detection, LOQ: Limit of quantitation

which, in turn, help in the establishment of degradation pathways and the intrinsic stability of drug molecule. To check the selectivity of the proposed method, degradation studies were carried out using acidic, alkaline, oxidative, photo, and thermal conditions.

Procedure for forced degradation studies

To determine, whether the analytical method and assay were stability indicating or not, torsemide active pharmaceutical ingredient (API) was stressed under various conditions to conduct forced degradation studies. Intentional degradation was attempted to stress conditions of acidic (0.1 N hydrochloric acid [HCl]), alkali (0.1 N NaOH), oxidative (0.3% H_2O_2 and 3% H_2O_2), photo (sunlight), and thermal (heated at 105°C) to evaluate the ability of the proposed method to separate torsemide from its degradation products. If reasonable degradation was seen under the above conditions, the testing can be stopped at this point. However, in case, no degradation was seen under the above conditions, the drug should be subjected to higher strengths and for longer duration. If total degradation was seen after subjecting the drug to initial conditions, the strength of acid/alkali/oxidative strength can be decreased to along with decrease in the reaction temperature.

Acid degradation

Accurately 2.5 mg of the torsemide pure drug was weighed and transferred into 25 ml clean dry volumetric flask. The drug was dissolved in small quantity of methanol and then volume was made up to the mark with 0.1 N HCl (100 µg/ml). The flask was kept aside for 15 min at room temperature. Periodically (0.15 min), 2 ml was taken in a 10 ml volumetric flask, add 5 ml of mobile phase and adjust the pH between 3 and 4 by adding 0.1 N NaOH, dilute with mobile phase up to the mark (20 µg/ml). The solution was injected under above chromatographic conditions and peak area was measured. The represented data are shown in Tables 14 and 15.

Alkaline degradation

Accurately 2.5 mg of the torsemide pure drug was weighed and transferred into 25 ml clean dry volumetric flask. The volume was made up to the mark with 0.1 N NaOH (100 µg/ml). The flask was kept aside for 15 min at room temperature. Periodically (0.15 min), 2 ml was taken in a 10 ml volumetric flask, add 5 ml of mobile phase and adjust the pH between 3 and 4 by adding 0.1 N HCl, dilute with mobile phase up to the mark (20 µg/ml). The solution was injected under above chromatographic conditions and peak area was measured. The represented data are shown in Tables 16 and 17.

Oxidative degradation

Accurately 2.5 mg of the torsemide pure drug transferred into 25 ml clean dry volumetric flask. The volume was made up to the mark with 3% H_2O_2 (100 µg/ml). The flask was kept aside for 5 days at room temperature. Periodically (0, 1, 24, 48, 72, 96, and 120 h), 2 ml was taken in 10 ml volumetric flask and made up to the mark with mobile phase (20 µg/ml). The solution was injected under above chromatographic conditions and peak area was measured. The represented data are shown in Table 18.

Photodegradation

Accurately 2.5 mg of the torsemide transferred into 25 ml clean dry volumetric flask. The drug was dissolved in small quantity of methanol and volume was made up to the mark with HPLC water (100 µg/ml). The flask was exposed to sunlight for 9 h. Periodically (0, 1, 3, 6, and 9 h), 2 ml was taken in 10 ml volumetric flask and made up to the mark with mobile phase (20 µg/ml). The solution was injected under the above chromatographic conditions and peak area was measured. The represented data are shown in Table 19.

Thermal degradation of solid sample at 105°C

Accurately 300 mg of the torsemide kept in Petri dish and maintained at a temperature of 105°C in a controlled temperature oven; periodically (0, 1, 3, 6, 9, and 12 h), 2.5 mg of sample was weighed and transferred into a 25 ml volumetric flask. The drug was dissolved in small quantity of methanol and volume was made up to the mark with HPLC water (100 µg/ml). From the above solution, 2 ml was taken in 10 ml volumetric flask and made up to the mark with mobile phase (20 µg/ml). The solution was injected under the above chromatographic conditions and peak area was measured. The represented data are shown in Table 20.

Table 12: Robustness studies of torsemide

Parameter	Conditions	R_t (min)	Area (n=3)	% assay	Remarks
Optimized	Methanol:water in ratio of 90:10 1 ml/min, λ_{max}: 287 nm	8.267	11,794,526±3042	100	-------
Flow rate	0.9 ml/min	8.850	12,040,495±2761	102.08	Not robust
	1.1 ml/min	7.547	12,583,910±7913	106.69	Not robust
Mobile phase	Methanol:water (88:12)	8.913	11,891,551±9327	100.82	Robust
	Methanol:water (92:8)	7.613	11,907,045±5575	100.95	Robust
Wavelength	285 nm	8.253	12,010,265±5548	101.82	Robust
	289 nm	8.220	11,785,983±9150	99.92	Robust

Table 13: System suitability parameters of torsemide

S. No.	Parameter	Values obtained	Acceptance criteria
1.	Retention time	8.267	----
2.	Theoretical plates	4706	>2000
3.	Peak asymmetry	1.07	≤1.5

Table 14: Acid degradation of torsemide in 0.1 N HCl

S. No.	Time (h)	Peak area	% degradation
1.	0 h	6,212,075	0
2.	15 min	4,541,054	26.89

HCL: Hydrochloric acid

Table 15: Degradants formed during acid degradation

S. No.	Degradants	Retention time (min)	Peak area
1.	1	**1.910**	**1,597,523**

Table 16: Alkaline degradation of torsemide (0.1 N NaOH)

S. No.	Time (h)	Peak area	% degradation
1.	0	8,010,462	0
2.	15 min	5,382,290	32.81

Table 17: Degradants formed during alkaline degradation

S. No.	Degradants	Retention time (min)	Peak area
1.	1	1.907	1,751,143

Table 18: Oxidative degradation of torsemide in 3% H_2O_2

S. No.	Time (h)	Peak area	% degradation
1.	0	11,024,387	0
2.	72	9,931,999	9.91
3.	120	9,098,462	17.46

Table 19: Photodegradation of torsemide

S. No.	Time (h)	Peak area	% degradation
1.	0	11,891,551	0
2.	6	10,535,474	11.40
3.	9	9,748,341	18.02

Table 20: Thermal degradation (at 105°C) of torsemide

S. No.	Time (h)	Peak area	% degradation
1.	0	12,722,511	0
2.	3	12,214,446	3.99
3.	6	11,700,324	8.03
4.	9	11,024,387	13.34
5.	12	9,398,462	26.12

RESULTS

Selection of wavelength

Wavelength for detection was selected by obtaining absorption spectra of torsemide in methanol using double-beam ultraviolet–visible spectrophotometer (Lab India). The complete spectrum of torsemide and its λ_{max} is shown in Fig. 6. It shows that torsemide has λ_{max} at 287 nm. The same wavelength was used in HPLC method development where the impurities can also be detected.

Several solvent systems were tried to get good optimized conditions for torsemide.

Specificity and selectivity

Specificity is the ability of a method to discriminate between the intended analyte(s) and other components in the sample. Selectivity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients.

Volume of 20 µl of working placebo sample solution was injected into the chromatograph and the chromatogram was recorded and presented below. No peaks were found at retention time of 8.267 min and drug was clearly separated from its degradants. Hence, the proposed method was specific and selective for the detection of torsemide.

The acid degradation was performed with 0.1 N HCl up to 15 min at room temperature.

The drug showed liability to acid hydrolysis at room temperature. It decomposed to an extent of 26.89% in 0.1 N HCl in 15 min.

The alkaline degradation was performed with 0.1 N NaOH up to 15 min at room temperature.

The torsemide showed liability to alkali hydrolysis at room temperature. It decomposed to an extent of 32.81% in 0.1 N NaOH in 15 min.

The oxidative degradation was first performed with 0.3% H_2O_2 for 7 days and degradation was not observed. Further, the oxidative degradation was performed with 3% H_2O_2 at room temperature up to 5 days.

The torsemide showed more liability to oxidative stress at room temperature. It decomposed to an extent of 9.91% in 3% H_2O_2 in 72 h and 17.46% in 120 h. The photodegradation was performed by placing the drug solution in sunlight for 9 h.

The drug is liable to photodegradation in sunlight. The drug decomposed at an extent of 11.40% in 6 h and the degradation increased to 18.02% in 9 h.

The thermal degradation was performed by placing the powdered drug sample in controlled oven at 105°C for 12 h.

The drug is liable to thermal degradation at a temperature of 105°C. The drug decomposed at an extent of 3.99% in 3 h and the degradation increased to 8.03% in 6 h and the extent of 13.34% degradation in 9 h and 26.12% degradation in 12 h.

DISCUSSION

The method was validated for all validation parameters as per the ICH guidelines. The linearity range of torsemide was 10–60 µg/ml. The value of correlation coefficient was 0.9993. The percentage RSD values in the precision studies were <2%. This confirmed that the method was sufficiently precise. The accuracy of the method was validated by recovery studies and was found to significant and under specification limits, with percentage recovery 100.56–101.03 (within acceptable range of 98–102%). The assay result was found to be 100.88% (i.e., within 95–105%). The method also passes the specifications for robustness parameters. LOD of torsemide was found to be 0.0162 µg/ml and LOQ of torsemide was found to be 0.0534 µg/ml.

Forced degradation HPLC method has been developed for the estimation of torsemide API. Forced degradation HPLC method was developed with mobile phase system of methanol:water in the ratio of 90:10 v/v. The flow rate of 1 ml/min was used on Inertsil ODS 3V column (250 mm×4.6 mm, 5 µm particle size). The retention time of torsemide was observed at 8.267 min. The drug was highly sensitive to alkali followed by liable to acid, photolytic, thermal, and oxidative conditions. The developed and validated stability-indicating HPLC method is found to be linear, precise, accurate, specific, and robust. Hence, the method can be used routinely for the estimation of torsemide API.

The APIs, namely, torsemide is the essential therapeutic agent in the treatment of hypertension. Among the analytical methods available in estimation and quantification, HPLC method is an emerging technique reliable in vast areas of interest that incited the author to undertake method development and validation as per the ICH guidelines for the same. Reverse-phase HPLC method development was done for torsemide using methanol and water in the ratio of 90:10 as mobile phase and detection was performed at 287 nm with a retention time of 8.267 min and peak asymmetry of 1.07.

The method was validated for all validation parameters as per the ICH guidelines. The linearity range for torsemide was 10–60 µg/ml, with r^2=0.9993. The percentage RSD for intra- and interday precision was <2%. The method has been validated in assay of tablet dosage forms. The accuracy of the method was validated by recovery studies and was found to be significant and under specification limits, with percentage recovery 100.56–101.03% (within acceptable range of 98–102%). The method was also passes the specifications for robustness parameters.

A stability study on torsemide was carried out and an efficient HPLC method for the quantification of torsemide. The results of stress testing of the bulk drug, undertaken according to the ICH guidelines, revealed that degradation was observed under acidic, alkaline, oxidizing, thermal, and photolytic conditions. The drug was highly sensitive to alkali followed by liable to acid, photolytic, thermal, and oxidative conditions.

CONCLUSION

In the present study, the API, namely, torsemide (sodium-potassium-chloride symporter inhibitors) is the essential therapeutic agent in the treatment of hypertension. HPLC method is an emerging technique reliable in vast areas of research that incited the author to undertake method development and validation as per the ICH guidelines for the same. The work was aimed comparatively to the earlier literature report in connection to the priority of the present investigation with respect to parameters such as linearity, assay, accuracy, precision, LOD, LOQ, and robustness.

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AUTHORS' CONTRIBUTIONS

We declare that this study was performed by the authors mentioned in this article and all liabilities relating to claims relating to the substance of this article will be borne by the authors. Mrs. K.V. Lalitha collected the data, analyzed the data, and performed all the laboratory works under the guidance of Raveendra Reddy J and Devanna N; also, they proofread the whole manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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