Original Article

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Development and validation of high-performance thin-layer chromatography and MS-MS method for estimation of terizidone in pharmaceutical dosage form

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ABSTRACT

Objective: Estimation of terizidone in pharmaceutical dosage form by using high-performance thin-layer chromatography (HPTLC) CAMAG system. **Materials and Methodology:** The separation was achieved using precoated silica gel 60 F_{254} aluminum plates with mobile phase toluene: n-Butanol 9:1 mL % v/v at wavelength 268 nm. **Results:** The linearity range was found between 50 and 300 ng/mL-band having correlation coefficient 0.997. The recovery study of method showed the recovery between 99.3% and 101.6%. The limit of detection (LOD) and limit of quantification (LOQ) values for method were 0.981 and 2.973, respectively. The R.S.D. of precision study was <2. The method parameters were validated as per the ICH guidelines. The forced degradation studies revealed that the drug found stable in acidic, thermal, and photodegradation. **Conclusion:** The liquid chromatography–mass spectrometry study of degraded products gave the idea about the fragmentation patterns of degraded products. The developed method can be used for routine analysis of terizidone and its formulations.

Keywords: High-performance thin layer chromatography, high-performance thin-layer chromatography, isolation, mass spectrometry/MS, terizidone, validation

INTRODUCTION

Terizidone is chemically named as 4,4'-{1,4-phenylenebis[methylylideneazanylylidene]} bis(1,2-oxazolidin-3-one) [Figure 1]. It acts as bacteriostatic in MDR-TB.[1] It prevents bacterial cell synthesis by competitively inhibiting L-alanine racemase and D-alanine ligase. The drug is administered in conjunction with second-line anti-TB drugs.[2,3]

In the literature, only two spectrophotometric methods are available for estimation of terizidone but no high-performance thin-layer chromatography (HPTLC) method has been reported.[4,5] HPLTC is the simplest, rapid, robust, and efficient tool in the quantitative analysis of compounds. This technique can work with trace quantities, can be used for multicomponent formulation if components have same absorbance maximum (λ_{max}) and technique is less prone to interferences while ultraviolet (UV) technique offers a clear, homogeneous sample solution and UV spectroscopy does not work with the components who deviate from Beer- Lambert's law, there are limitations for high concentrations of analytes also. Therefore, there is a need to develop an analytical method which can quantify the drug by the simple, easy, and accurate way.

The aim of the work was to develop and validate an HPTLC method for estimation of terizidone in the pharmaceutical dosage form. The parameters of methods were validated according to ICH guidelines.[6]

MATERIALS AND METHODOLOGY

Materials

Chemicals

The pure terizidone sample was gifted by Macleods Pharmaceuticals Ltd, Gujrat. Analytical gradetoluene and

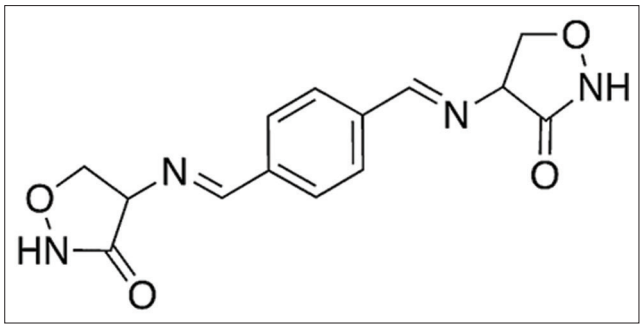


Figure 1: Chemical structure of terizidone

n-butanol were purchased from Loba Chemie. Methanol was used was of analytical grade.

Instrumentation

The HPTLC system used for method development of CAMAG system which consisted Linomat 5 sample applicator, CAMAG twin trough chamber, TLC scanner. Pre-coated aluminum plates (size 10 cm × 10 cm) coated with Silicagel 60 F_{254} (63–200 μm) were used for application. Syringe of 100 μL capacity was used for injections.

Methodology

Preparation of solutions

The stock solution was prepared by weighing 10.0 mg of pure terizidone transferring into 10.0 mL. volumetric flask. Methanol 5.0 mL was added and sonicated for 10 min, and volume made up to the mark by addition of methanol. From above solution, 50 mL solution was transferred to 10.0 mL. volumetric flask and volume made up to the mark by addition of methanol.

Standard solution of terizidone was prepared by pipetting out 0.4 mL. of stock solution and transferred to 10.0 mL volumetric flask. Volume was made up to the mark with methanol.

The sample solution was prepared by weighing and crushing 20 tablets into fine powder. Tablet powder equivalent to 10.0 mg of terizidone was transferred to 10.0 mL volumetric flask, and 5.0 mL methanol was added. After 10 min of sonication, the volume was made up to the mark by methanol. The solution was filtered through Whatman paper Grade 1 (pore size 11 μm) to obtain clear solution. From above solution, 5.0 mL was pipetted out and transferred to 10.0 mL volumetric flask, volume was made up to the mark with methanol.

From above solution, 0.4 mL solution was transferred to 10.0 mL volumetric flask and volume were made with methanol.

Mobile phase selection and calibration curve

Aliquot portions of the standard stock solution, 2 μL each, were applied in the form of band (4 mm) on pre-activated TLC plate and plates were run using the different solvent system in an attempt to achieve desired R_f value (0.2–0.8).

After development, the plate was allowed to dry and scanned under 200–700 nm UV range. The maximum absorbance was found at 268 nm. The absorbance spectra at 268 nm have shown in Figure 2.

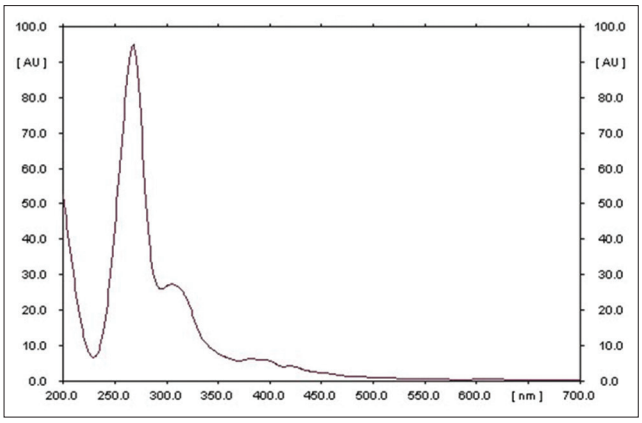


Figure 2: Absorbance spectra at 200–700 nm

Calibration curve of terizidone was done by applying 6 bands of standard solution of volume ranging from 0.1 to 0.6 mL. (concentration range 50–300 ng/mL – band, respectively). Peak areas were measured against concentration. Graph of concentration versus peak area was plotted and linearity equation was found out 6 bands of standard were applied on one plate (0.1–0.6 mL); however, each standard was injected in triplicate.

Analysis of standard laboratory mixture and marketed formulation

Standard solution was analyzed and percent amount of drug was recovered**.** Six bands were applied on one plate (n=6) and **%** R**.**S**.**D was calculated. Sample solutions were prepared as the procedure given above and amount per tablet was estimated and **%** label claim was calculated**.** The number of analysis was repeated for 6 times (n=6)**.** The R**.**S**.**D**.** was calculated**.**

Method validation

Method validation was carried out as per the ICH guidelines of analytical method validation (Q_2R_1).[7-9]

Precision

Precision of method was done as system precision and method precision.

System precision was carried out by applying six bands from same standard solution with respect to check the repeatability of the system. System precision was done to check whether applicator applied the same volume of sample at every application, i.e., 2 μL.

However, method precision was done to check the reproducibility of the system, i.e., every time, whether system was able to recover the applied amount of drug. It was done by applying 6 bands from 6 different sample solutions, and amount of drug estimated was calculated.

Accuracy

The accuracy studies were conducted at three levels, i.e. 80**%**, 100**%,** and 120**%.** It was done by weighing tablet powder

equivalent to 10.0 mg of terizidone and transferring them to 9 different 10.0 mL volumetric flasks and adding 8.0 mg, 10 mg, and 12 mg of pure drug in 9 flasks in triplicate form. Solutions were done as per the procedure described in sample solution preparation, and amount of drug recovered was calculated.

Robustness

The robustness studies were carried out by doing small but deliberate changes in saturation time, mobile phase composition, total mobile phase, and time from development to scanning. The R.S.D. was calculated for every factor.

LOD and LOQ

The sensitivity of the system was determined by calculating LOD and LOQ. It should not be more than 3 for LOD and not more than 10 for LOQ.

Forced Degradation Study

The stability of the method was determined by exposing the drug to different stressed conditions such as acidic, basic, oxide, thermal, neutral, and photodegradation.

Six different samples were prepared by weighing 10.0 mg of pure drug and transferring to six different 10.0 mL volumetric flasks. Then, 3 mL of 0.1 M HCl, 0.1 M NaOH, and 3% H_2O_2 and distilled water were added to each flask in the first four flasks, respectively. The solution was refluxed in water bath at 80°C for 3 h. Pure drug sample was heated to 60°C for 3 h. and for photodegradation study, pure drug sample was heated at 60°C and exposed to UV light for 24 h. The samples exposed to heat and sample exposed to UV light were transferred to the 5th and 6th flask, respectively. Solutions were made as per procedure described in sample preparation. The % assay of active substance was calculated.

Isolation and identification of degradation products by HPTLC-MS/MS

Isolation of degraded products was done by applying degraded sample solution on TLC plates (9 μL per band) and applying as band and plate was developed under optimized chromatographic conditions. After drying the plate, it was observed under UV cabinet and degraded band was identified and marked. The band was then scrapped and soaked overnight in methanol. On next day, the sample was filtered through Whatmann filter paper Grade 1 (pore size 11 μm) and subjected to MS/MS. After recording of MS/MS spectra, IR spectra was taken and identification of fragmentation patterns were done by observing IR spectra and MS/MS spectra.[10,11]

RESULTS AND DISCUSSION

After trying several permutations and combinations, it was found that the combination of toluene and n-butanol in ratio 9:1% v/v gave the compact band for terizidone at R_f value of 0.60 ± 0.03, as shown in Figure 3.

Linearity and Calibration Curve

The increase in area was directly proportional to concentration. The linearity curve was found to be linear and regression coefficient was found 0.997 with equation y = 9.535x + 1533. The linearity curve is shown in Figuer 4.

Analysis of Bulk Drug and Marketed Formulations

The % assay of the active substance in standard laboratory mixture as well as in marketed formulations was calculated. The % R.S.D. was found to be <2. The results of analysis of the substance in the standard mixture and in the pharmaceutical formulation are shown in Table 1.

Accuracy

The accuracy studies were carried out to check whether the pure drug can be recovered from tablet solution of the terizidone. The study was carried out at three levels, i.e., 80%, 100%, and 120%. The results showed that the percent recovery was between ranges 99.3–101.6%. As the results are shown in Table 2, the R.S.D. was found to be 0.59.

Table 1: Results of bulk drug and marketed formulation

Mean % estimation (n=6)	S.D. (±)	R.S.D. (R≤2)
Analysis of bulk drug		
100.2	1.62	1.6
Analysis of marketed formulation		
251 mg/tab	1.15	1.1

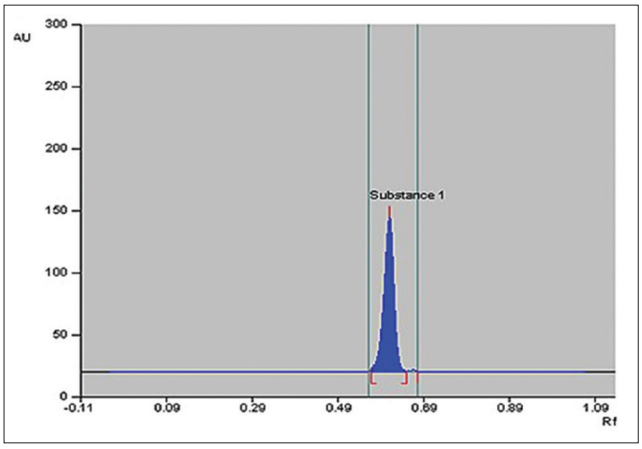


Figure 3: Typical densitogram of terizidone (0.60 ± 0.03)

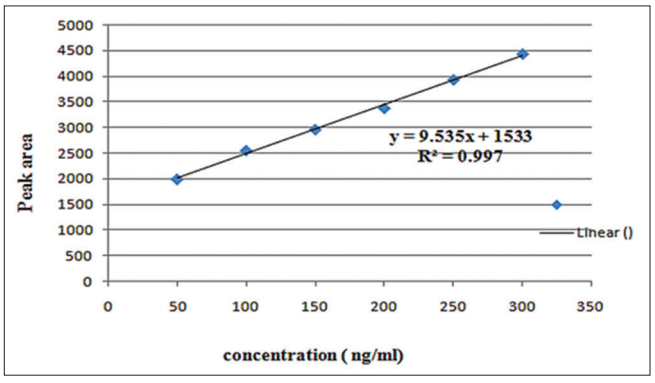


Figure 4: Calibration curve of terizidone

Robustness

The robustness study was done by doing some deliberate, but small changes in the parameters of optimum condition to check whether it affects the results. Table 3 explains the results of robustness study.

LOD and LOQ

The LOD and LOQ of the method were found to be 0.981 μg/mL and 2.973 μg/mL, respectively.

Forced Degradation Study

The stability of the method was checked by doing forced degradation study and percent assay of active substance was calculated. The drug solution was subjected to different stress conditions and analyzed. Terizidone was degraded in acidic condition [Figure 5a] showing two peaks of R_f values 0.01 (1), 0.02 (2), 0.25 (3) in which figures in bracket were indicated as peak number, however, in oxidative condition [Figure 5b], 5 peaks were found having R_f values 0.26 (1), 0.36 (2), 0.40 (3), 0.43 (4), and 0.80 (6) and photo degradation condition [Figure 5c] showing 4 peaks of R_f values 0.50 (1), 0.55 (2), 0.80 (4), 0.88 (5). The assay of active substance in acidic, oxidative and photodegradation was found to be 81.05%, 81.40%, and 93.25%, respectively. Degradation found the maximum in neutral condition and minimum in thermal condition [Figure 5d]. The results of % assay of active substances and R_f of degraded products are shown in Table 4, while densitograms of different stressed conditions are shown in Figure 5a-d.

Table 2: Results of recovery studies

Level	Mean % recovery	R.S.D. of mean % recovery
80	99.6	
100	100.5	0.59
120	100.8	

Table 3: Results of robustness study

Factor	(±) S.D. for peak area
Saturation time (± 5 min)	1.5
Total mobile phase (± 1 mL)	1.6
Mobile phase composition (± 0.1 mL)	1.1
Time from development to scanning (± min)	1.6

Identification of Degraded Products by IR Spectroscopy And Tandem Mass Spectroscopy (MS)

In the study, the samples treated with UV degradation were assigned as Photo-1, whereas the samples treated with distilled water as Hydro-1. In tandem MS, Q1 and Q3 scan were done which are qualitative scans, in Q1 scan, parent ions of single m/z ratio were selected and passed to Q2 collision cell where ions were collided with argon gas to form product ions. Further productions of single m/z ratio were passed in the Q3 scan to obtain spectra. The MS spectra are shown in Figures 6-9, the Q1 and Q3 spectra for Photo-1, Q1 and Q3 spectra for Hydro-1, respectively.

Table 4: Results of forced degradation study

Stress condition	Temperature and time	% assay of active substance	R_f of degraded products
Acid (0.1 M HCl)	80°C for 3 h	81.05	0.25,0.78
Alkali (0.1 M NaOH)	80°C for 3 h	82.89	0.01, 0.04, 0.08, 0.28
Oxide 3% H_2O_2	80°C for 3 h	81.40	0.30, 0.36, 0.40, 0.43, 0.81
Neutral	80°C for 3 h	80.4	0.01, 0.05, 0.09, 0.30, 0.85
UV degradation	60°C 24 h	93.25	0.50, 0.55, 0.8, 0.88
Thermal	60°C for 24 h	94.82	0.51, 0.80

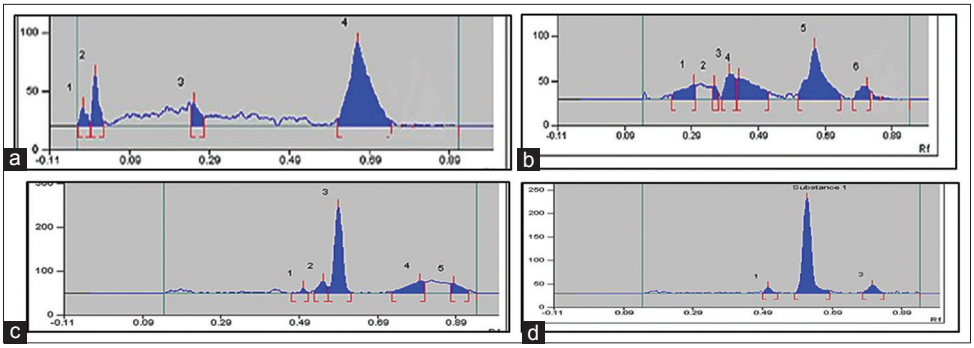


Figure 5: (a) Densitogram of degraded sample treated with 0.1 M HCl (No. of peaks: 03 at 0.01 (1), 0.02 (2), 0.25 (3), (b) densitogram of degraded sample treated with 3% H_2O_2 (No. of peaks: 05 at 0.26 (1), 0.36 (2), 0.40 (3), 0.43 (4), and 0.80 (6), (c) densitogram of degraded sample treated with ultraviolet light (No. of peaks: 04 at 0.50 (1), 0.55 (2), 0.80 (4), 0.88 (5). (d) Densitogram of the degraded sample on thermal treatment (No. of peaks: 02 at 0.51 (1), 0.80 (3); substance 1 referred to desitogram of pure drug terizidone

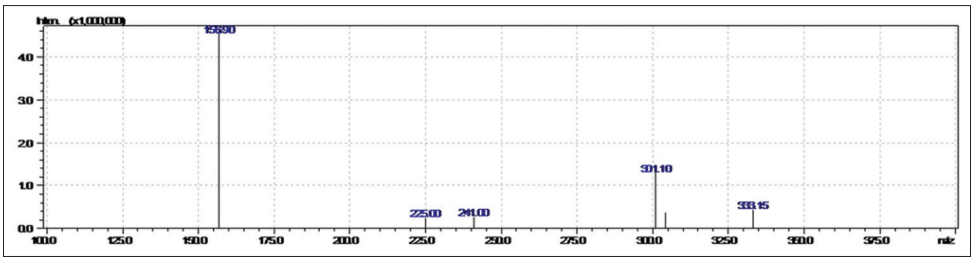


Figure 6: Q1 spectra for Photo-1

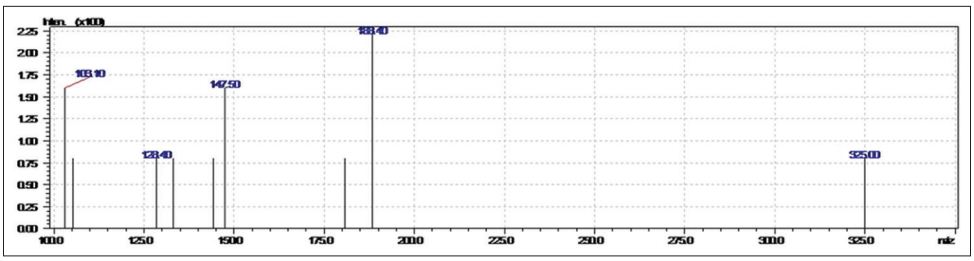


Figure 7: Q3 spectra for Photo-1

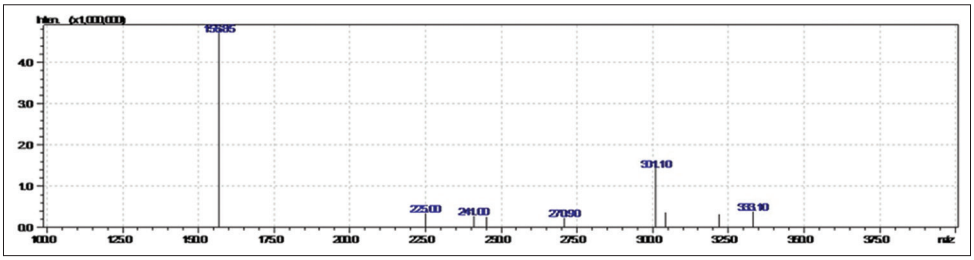


Figure 8: Q1 spectra for Hydro-1

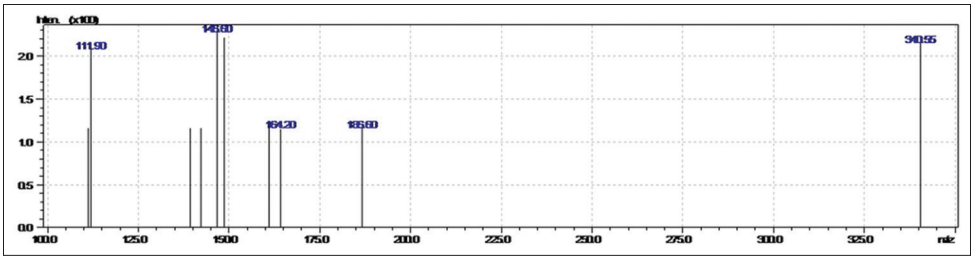


Figure 9: Q3 spectra for Hydro-1

Isolation and identification of degradation product of terizidone, Photo-1

Isolation of photodegraded product (Photo-1) was carried out using a procedure which is mentioned above. This Photo-1 was identified by using MS-MS.

Identification of Photo-1 structure by tandem MS

Chemically terizidone is 4,4'-{1,4-phenylenebis[methylylidene azanylylidene]}bis(1,2-oxazolidin-3-one) which has empirical formula $C_{14}H_{14}N_4O_4$, and the molecular weight is of about 302.2 g/mol. When this drug is allowed to degrade under the UV degradation it was degraded to form Photo-1, it gets degraded by the molecular weight of about 156.9 g/mol. This structure of the degraded product was predicted by loss in molecular weight of the Photo-1 in comparison to the pure drug. During neutral degradation of terizidone bond was broken down and degraded to 2-[(1-pent-1-en-1-yl] cyclohex-1-en-3-yne. This ring had a molecular weight of about 146 g/mol.

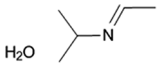
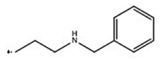
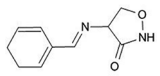
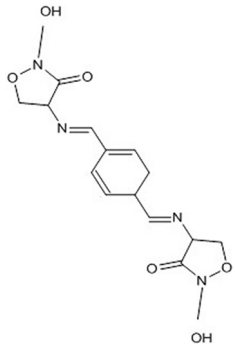
When MS spectra of this Photo-1 were recorded it show major four molecular ion peaks of different m/z ratio as shown in Table 5.

Proposed mass fragmentation pattern of the hypothetical mass fragmentation pattern Photo-1 for photolyzed drugs on the basis of MRM transitions has been incorporated, and that helps in confirmation of structure on the basis of diagnostic ions. After recording Q1 spectra of Photo-1, 301.1 was selected as precursor ion and further fragmented it, Q3 spectra were recorded, following fragmentation patterns were predicted [Table 6].

Table 5: molecular ion peak of different m/z ratio recorded in Q1 scan of Photo-1

Degradation peak name	m/z ratio
Photo-1	156.9
Photo-2	225.0
Photo-3	241.0
Photo-4	333.15

Table 6: Predicted fragmentation patterns from Q3 spectra for Photo-1

Analyte attached	Predicted structure	Exact mass	m/z (observed)
$C_5H_{13}NO$		103.10	103.10
$C_{11}H_{13N}{}^{\cdot+}$		147.10	147.20
$C_{10}H_{10}N_2O_2$		190.0	188.4
$C_{14}H_{14}N_4O_6$		334.0	333.3

Isolation and identification of degraded product Hydro-1

Isolation of hydrolyzed degraded product (Hydro-1) was carried out by using a procedure which is mentioned above. This Hydro-1 was identified using IR and MS-MS. IR spectra of pure drug and Hydro-1 was recorded, and major functional group and there stretching and bending wavelength was used to predict the structure.

Identification of Hydro-1 structure by tandem MS

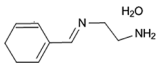
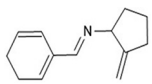
Chemically terizidone is 4,4'-{1,4-Phenylenebis[methylylid eneazanylylidene]}bis(1,2-oxazolidin-3-one) which has empirical formula $C_{14}H_{14}N_4O_4$, and the molecular weight is of about 302.2 g/mol. When this drug is allowed to degrade under the neutral degradation it was degraded to form Hydro-1, it gets degraded to the molecular weight of about 156.9 g/mol. This structure of the degraded product was predicted on the basis of loss in molecular weight of the Hydro-1 in comparison to the pure drug. During neutral degradation of terizidonebond was broken down and degraded to 2-[(1-pent-1-en-1-yl]cyclohex-1-en-3-yne.

This ring had a molecular weight of about 146 g/mol. When MS spectra of this Hydro-1 was recorded it show major five molecular ion peak of different m/z ratio as shown in Table 7.

Table 7: Molecular ion peak of different m/z ratio recorded in Q1 scan of MS

Degradation peak name	m/z ratio
Hydro-1	156.90
Hydro-2	225.1
Hydro-3	241.1
Hydro-4	270.90
Hydro-5	333.10

Table 8: Predicted fragmentation patterns from Q3 spectra for Hydro-1

Analyte attached	Predicted structure	Exact mass	m/z (observed)
C_8H_{16}		112.10	111.90
$C_9H_{14}N_2O$		166.11	164.20
$C_{13}H_{15}N$		185.2	186.6

Proposed mass fragmentation pattern of the hypothetical mass fragmentation pattern Hydro-1 for photolyzed drugs by MRM transitions has been incorporated, and that helps in confirmation of structure on the basis of diagnostic ions.

After recording Q1 spectra of Hydro-1, 301.1 was selected as precursor ion and further fragmented it, Q3 spectra were recorded, following fragmentation patterns were predicted [Table 8].

CONCLUSION

The simple HPTLC method was developed for estimation of terizidone in the pharmaceutical dosage form. The method was found accurate, precise. The method was validated as per ICH Q_2R_1 guidelines. The method shows linearity toward concentration with respect of peak areas, however, accuracy and precision of the method were able to product reproducible results. The drug solution was found stable under thermal and UV light degradation conditions. The liquid chromatography-MS (LC-MS/MS) study produced fragmentation patterns of degradation products (Photo-1 and Hydro-1), and it helped to predict fragmentation structures of both the products. The method can be able to produce reproducible results, so can be employed for routine analysis of terizidone in bulk and in pharmaceutical dosage forms.

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