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A stability-indicating HPLC method for estimation of doxylamine succinate in tablets and characterization of its major alkaline stress degradation product

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Abstract

Background: A new selective rapid RP-HPLC-DAD method was developed and evaluated for the quantification of doxylamine succinate (DOX) in bulk and pharmaceutical dosage form. The separation of DOX at different degradation conditions was achieved with a Kromasil C_{18} (4.6 × 250 mm, 5-μm particle size). The mobile phase employed comprised of phosphate buffer (pH 3.5) and methanol in the ratio of 45:55 v/v. The flow rate was kept maintained at 1.0 ml/min and eluents were detected at 262 nm. The drug was subjected to different stress conditions like acid, base, neutral, hydrolysis, oxidation, photolysis, and thermal degradation. The analytical performance of the proposed HPLC method was thoroughly validated in terms of linearity, precision, accuracy, specificity, robustness, detection, and quantification limits.

Results: The method produces linear responses that were found in the range of 10–50 μg/ml. The regression equation was found to be Y = 42984x − 10260. The correlation coefficient was found to be 0.9998. The LOD and LOQ for DOX were found to be 0.96 and 3.28 μg/ml, respectively. The short-term solution stability of DOX (100 μg/ml) was evaluated under (25 ± 2°C) storage condition and found to be 98.82 to 101%. The percentage recovery for DOX was in the range of 99.73 to 99.91%. The obtained results of the stress degradation study and peak purity data indicate the potential of the developed HPLC method to resolve degradants from DOX peak. The major alkaline degradation product was isolated using preparative chromatographic technique and extensive FT-IR was performed to ascertain the structure of the alkaline degradant.

Conclusion: It was concluded that the proposed method was simple, sensitive, accurate, cost-effective, and less time-consuming for the quantification of DOX. This method was successfully utilized for stability testing of commercially available DOX tablets. Hence, the proposed method can be applied for routine quality control of DOX in bulk drug as well as in marketed formulations.

Keywords: Doxylamine succinate, Stress study, RP-HPLC, Diode array detection, ICH

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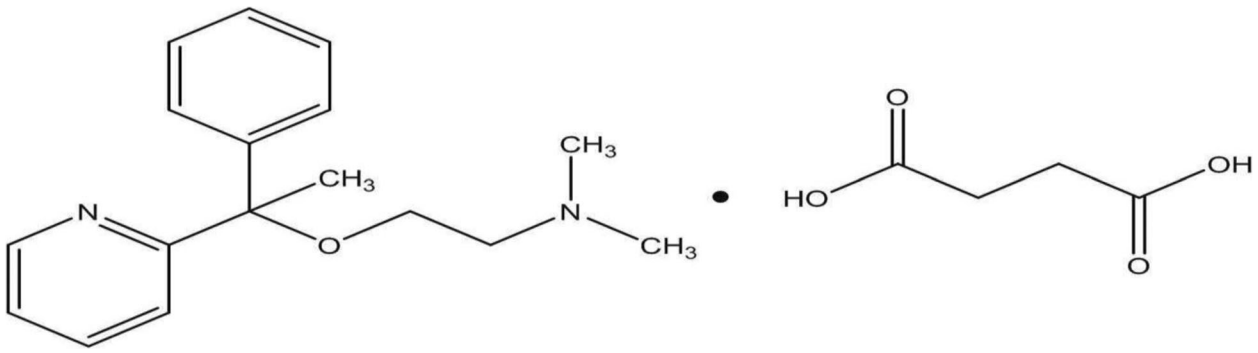

Fig. 1 Chemical structure of doxylamine succinate

Background

Doxylamine succinate (DOX), (RS)-N,N-dimethyl-2-(1-phenyl-1-pyridin-2-yl-ethoxy)-ethanamine (Fig. 1), is chemically used as an antihistaminic drug and antimuscarinic agent [1, 2]. It exhibits moderate alkaline nature with a pKa of 8.8; apart from this, it is commercialized as succinate salt is one of the first-generation H_1 receptor antagonists of the ethanolamine group [3–5]. It belongs to counter sedatives to treat insomnia having decongestants and antitussives to mitigate symptoms of cough and cold in a patient. The combination of DOX and pyridoxine approved by the American College of Obstetricians and Gynecologists for administration was recognized for the treatment of nausea and vomiting [6–8]. Usually, for product advancement, stability examination plays a crucial part; moreover, product performance and its quality mainly depend upon various factors such as temperature, pH, humidity, and light. It also facilitates authorized shelf life's suggested storage condition. For drug product development, active drug and product degradant play a significant role [9–11]. However, there is a growing concern against the progress of stress testing using stability-indicating assay method as this assimilated in International Conference on Harmonization (ICH) guidelines. This path is being used in the amalgamation of drugs useful in the existence of degradant products [12]. As previously described, methods such as UV spectrophotometric [13–16], HPLC [17–21], HPTLC [22], GC [23], FT-IR [24, 25], and capillary electrophoresis [26] were only reported

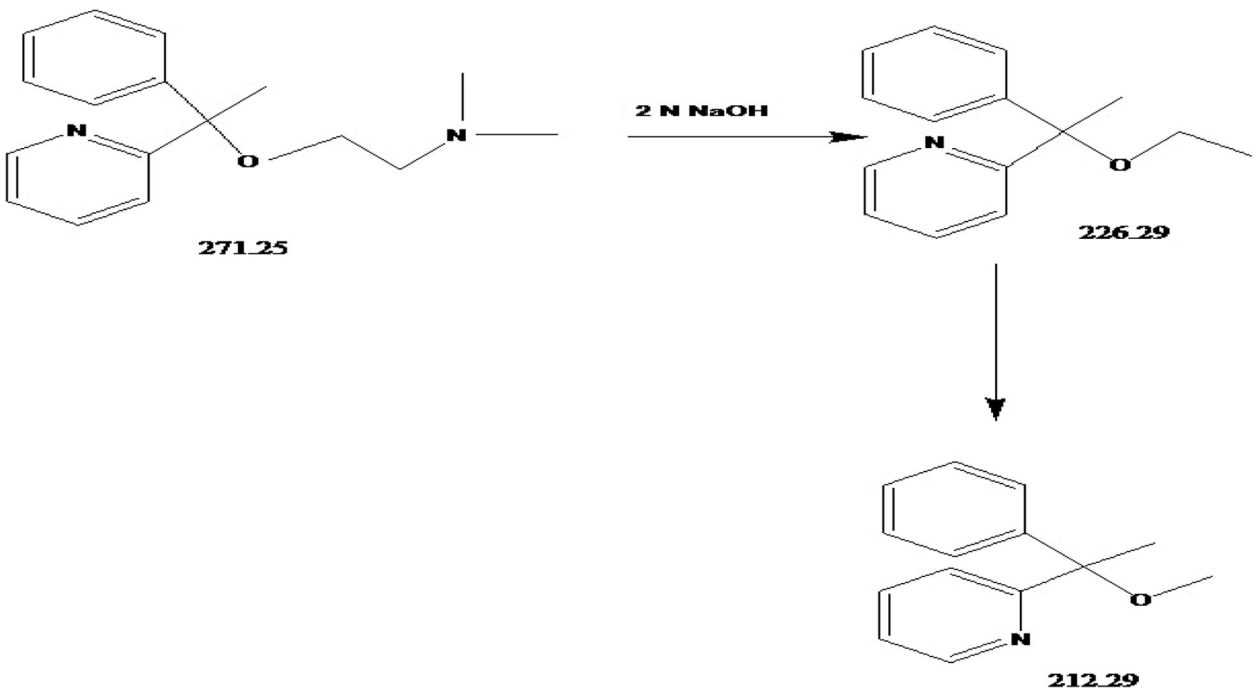

Fig. 2 Scheme of alkaline degradation of DOX

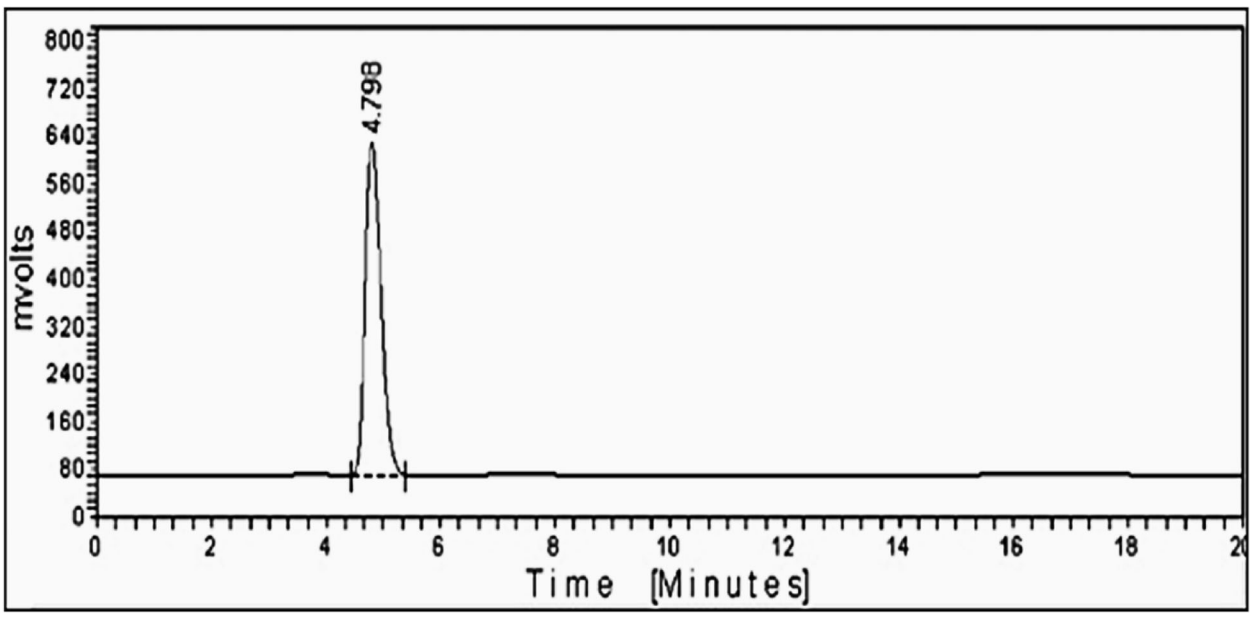

Fig. 3 Typical chromatogram

for estimation of DOX individually [27] or else in combination with another drug [28, 29]. According to reported references to the best of our knowledge, no single method against stress degradation was disclosed using RP-HPLC-DAD; hence, the current intent includes (i) quantitative determination of DOX in the presence of degradation product by the RP-HPLC-DAD method, (ii) carrying out the stress studies on DOX under the ICH-stated conditions, (iii) isolation of degradation products by preparative chromatographic technique, and (iv) characterization and establishment of the degradation pathway of degradation products.

Methods

Working standards of pharmaceutical-grade DOX were obtained as a generous gift from Cipla Pharmaceuticals Pvt. Ltd., Mumbai, India. The purity of DOX was found to be 99.9% on a dried basis. Restavit tablet label claim: each tablet contains 25 mg of DOX used for the study. Sodium hydroxide, hydrochloric acid, and hydrogen peroxide were purchased from Qualigens Fine Chemicals (Glaxo Ltd.) for the study. HPLC grade acetonitrile, water, phosphate buffer, and methanol were used in the study and purchased from Merck (Darmstadt, Germany).

Instruments

The modular HPLC system is equipped with Waters 510 HPLC pump (Waters Chromatography Division, Milford, MA, USA), a Rheodyne injector (20 μl), and solvent degasser with PDA 6000 LP detector. The chromatographic partition was accomplished on a Kromasil C_{18}

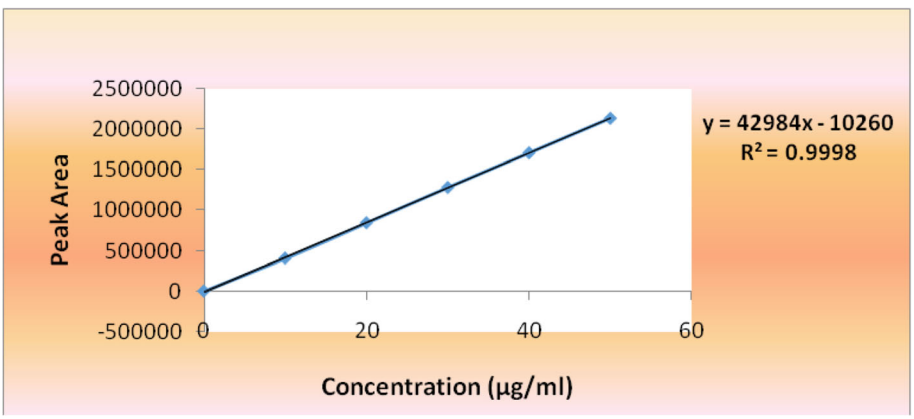

Fig. 4 Standard calibration curve of DOX

Table 1 Results of Cochran's C test

Concentration (ng/band)	S. D.*	Square of S. D.	Sum of the square of S. D.	C[a]
10	20.22	409		
20	97.15	9439		
40	91.69	8408	1490	0.498
50	17.07	2996		

S. D. standard deviation of three replications, *C*[a] Cochran's C value. *Average of three determinations

(250 mm × 4.6 mm and 5-μm particle size) column. The eluent was monitored using a photodiode array (PDA) detector. The ultrasonic bath (Biomedica, India) was used for the extraction of the drug from the tablets. A precision water bath equipped with an MV controller (i-therm, Biomedica, India) was used to execute preferred reactions in solution over stress degradation study. A thermal stability study was implemented in a dry air oven (Biotechnics BTI–20D, Mumbai, India). Other equipments used were sonicator (Biomedica, India), analytical balance (Shimadzu AUX 220, Japan), and auto pipettes (Eppendorf, Hamburg, Germany).

Preparation of standard solution

To prepare a stock solution of DOX, weigh exactly 50 mg of DOX and transfer into a separate 50-ml volumetric flask containing 25 ml of mobile phase with continuous stirring. The retrieved transparent solution was ultrasonicated for 10 min after that volume was made up with the remaining mobile phase to get a stock solution of 1000 μg/ml. From this stock solution, sample aliquots (0.1, 0.2, 0.3, 0.4, 0.5 ml) were withdrawn and diluted in a 10-ml volumetric flask separately and made up the volume up to the mark with mobile phase to achieve the final concentration (10, 20, 30, 40, and 50 μg/ml) for DOX. All the volumetric flask used for analysis was covered with an aluminum foil against light preservation. Furthermore, the solution was kept at 4°C for 1-week condition and found stable. Additionally, the marketed formulation was transferred to a 50-ml volumetric flask containing a small volume of mobile phase (10 ml) and stirred to extract out drug; further volume of the flask was made up with mobile phase to get a final concentration of 500 μg/ml. Subsequently, from the groomed stock solution, aliquots (0.2, 0.4, 0.6, 0.8, and 1 ml) were withdrawn and transferred to a 10-ml volumetric flask to attain a closing concentration.

Table 2 Result of LOD and LOQ data

Drug	
DOX	
Limit of detection (μg/ml)	**0.96**
Limit of quantification (μg/ml)	**3.28**

Average of six determinations

Table 3 Intraday precision data

Drug	Concentration* (μg/ml)	Peak area	S. D.	C. V.
DOX	20	758601	± 1510	0.0998
	25	1198543	± 4964	0.2624
	30	1486948	± 5878	0.2597

S. D. standard deviation, *C. V.* coefficient of variance. *Mean of six determinations

Forced degradation studies on API and marketed formulation

A stress degradation study of marketed formulation and API was executed to arbitrate the stability of the developed method under different conditions using placebo and blank samples. All stress experiments were performed at 25-μg/ml concentration subjected to suitable dilution at periodical withdrawn followed by filtration of the sample before injection in the chromatographic system.

Degradation study

For acid, alkali, neutral oxidation, 25 μg/ml analyte in combination with methanolic solution, 2 M hydrochloric acid, and 2 M NaOH; water; and 6% H_2O_2 (20:80) were prepared in three replicates and solution reflux for 8 h at 70°C.

Photolytic degradation

For photolytic degradation analysis, 50 mg marketed formulation (tablets) and API were transferred in a petri dish and kept in a UV cabinet by exposing the intense UV radiation of shorter and longer wavelength for 48 h. After the closure time interval, stressed samples were diluted with mobile phase to obtain a final concentration of 25 μg/ml and then a volume of 20-μl solution was injected into the system.

Development of the analytical method

A chromatographic separation of API and tablet formulation from its degradation products was achieved using an isocratic mobile phase consisting of phosphate buffer (pH 3.5) and methanol in the ratio 45:55 v/v followed by filtration (0.45-μm nylon) and degassed before use. All determination at 20-μl volume with constant 1-ml flow

Table 4 Interday precision data

Drug	Concentration* (μg/ml)	Peak area	S. D.	C. V.
DOX	20	749543	± 2005	0.1324
	25	1182494	± 6712	0.356
	30	1442863	± 4724	0.209

S. D. standard deviation, *C. V.* coefficient of variance. *Mean of six determinations

Table 5 Results of the accuracy study

Level of recovery (%)	Theoretical concentration (μg/ml)		% Recovery
	Formulation	Pure drug	DOX
80	30	24	99.4
	30	24	100
	30	24	99.8
100	30	24	99.84
	30	24	99.68
	30	24	100.16
120	30	24	100.13
	30	24	99.73
	30	24	99.86

rate was performed and eluents were measured using a PDA detector at 262 nm.

Assay

Twenty tablets were weighed and tablet powder equivalent to 12.5 mg of DOX was diluted with 15ml of mobile phase in a volumetric flask and sonicated for 20 min for complete dissolution. Then, the volume was made up to 50 ml after filtration. Further, the 1-ml filtrate was diluted to 10ml with mobile phase in a volumetric flask and then filtered through a 0.45-μm nylon filter to yield the final concentration (25 μg/ml).

Isolation of alkaline degradation product

A weight equivalent to 100 mg of DOX was transferred to a volumetric flask containing 20 ml methanol and stir to get a clear solution. The final volume of 100 ml was achieved by subsequent addition of 2.0 M NaOH. The reflux assembly of the round bottom flask containing the resultant mixture was maintained at respective temperature along with a precision-controlled water bath at 70°C for 5 h. Under the alkaline set degradation condition, DOX was studied and evaluated by the proposed method wherein the majority of alkaline degradant isolated employing preparative column chromatographic technique. The scheme of qualitative estimation of DOX and alkaline degradant is accomplished under the alkaline stress condition shown in Fig. 2.

Table 6 Statistical validation for accuracy study

Level of recovery (%)	% Mean recovery[a]	Standard deviation	% RSD	S.E
80	99.73	± 0.305	0.305	0.176
100	99.89	± 0.244	0.244	0.141
120	99.91	± 0.203	0.230	0.117

Mean of three determinations

Table 7 Robustness study for the HPLC method

Factors	Chromatographic changes		
Flow rate (ml/min)	*Level*	*Retention time*	*Tailing factor*
0.9	−0.1	4.77	1.14
1.0	0	4.79	1.15
1.1	+ 0.1	4.78	1.16
	Mean	4.78	1.15
	S. D.	± 0.010	± 0.010
Mobile phase (v/v/v)			
44:56	−1.0	4.77	1.16
45:55	0	4.79	1.15
46:54	+ 1.0	4.83	1.17
	Mean	4.79	1.16
	S. D.	± 0.030	± 0.010
Wavelength (nm)			
261	−1.0	4.79	1.14
262	0	4.79	1.15
263	+ 1.0	4.78	1.15
	Mean	4.78	1.14
	S. D.	± 0.005	± 0.005

Validation of the developed analytical method

The recommended analytical approach was validated conferred to ICH guidelines. The system was checked for any obstructive substance or backdrop noises at the retention time of DOX found establish to a tolerable range of < 20%. A standard linearity plot was constructed by making the appropriate concentration of 10–50 μg/ml range. The precision study was performed with six replicate injections of the same concentration considering system error as the parameter of the precision experiment. All the retrieved findings were revealed as percent relative standard deviation (% RSD). The interday and intraday experiment was executed for three replicates for the same and on a different day. The accuracy of the current method was assessed through fortifying marketed formulation of three concentrations. Later, to estimate method's specificity, the resolution factor amidst peak area and nearest resolving peaks were

Table 8 Results of the analysis of tablet formulation of DOX

Sr. no.	Tablet strength 50 mg		
	Weight of tablet powder taken (mg)	Amount of drug estimated (mg/tablet)	% Label claim
1	69.0	24.97	99.88
2	69.0	25.05	100.2
3	69.0	24.93	99.72
4	69.0	24.88	99.52
5	69.0	24.96	99.84
6	69.0	25.01	100.04

Table 9 Statistical validation of analysis of tablet formulation

Sr. no.	Drug	Amount of drug estimated (mg/tab)	% Label claim	S. D.	C. V.	S.E
1	DOX	24.96	99.86	± 0.2382	0.2385	0.0972

Mean of six determinations

Table 10 Result of the solution stability study of DOX

Sr. no	Time	Amount of drug estimated DOX*	% Label claim	S. D. (±)	C. V.
1	30 min	24.70	98.82	0.619	0.623
2	1 h	24.98	99.92	0.346	0.350
3	2 h	25.00	100.0	0.424	0.427
4	4 h	24.75	99.00	0.537	0.541
5	8 h	24.88	99.52	0.801	0.804
6	24 h	25.25	101.0	0.599	0.603

*Average of three determinations

considered. The peak purity of the entire peaks along with degradant using diode array (DAD) detector was checked for its selectivity (www.ich.org/fileadmin/public_web_site/ich_products/guidelines/quality/q1a_r2/step4/q1a_r2_guideline.pdf).

Result

Optimization of experimental condition

The recommended RP-HPLC method was practiced for the imperative separation of intended degradation product; varied blend and fractions were tried as mobile phase. The mobile phase in combination with phosphate buffer (pH 3.5) and methanol (45:55 v/v) was used and found a sharp well-resolved symmetrical peak at a 1-ml/min flow rate of the sample. The DOX chromatogram is retrieved at 4.79 min as represented in Fig. 3. The invented composition of the mobile phase performs together with degraded sample solution and degraded formulation.

Validation of the developed stability-indicating method

The analytical method was validated with respect to parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery, and robustness.

Linearity

A linear response was found in the range of 10–50 μg/ml. The series of five-point linear curve (Fig. 4) were plotted with obtained peak area against concentration followed by 0.9998 correlation coefficient with % RSD in the range of 0.1–0.9. The typical value of the regression equation was found to be $Y = 42984x - 10260$. Cochran's C value was found to be < 0.725 indicating the good linearity of DOX. The obtained results are summarized in Table 1.

LOD and LOQ

The developed method of DOX was calculated for LOD and LOQ using calibration curves along with the slope of linearity using formula $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$ (where σ = standard deviation of response and S = slope of calibration curve). The LOD and LOQ for DOX

Table 11 Mass balance and peak purity study of DOX

Stress conditions	% Degradation*	Purity angle	Purity threshold	% Assay	Mass balance	RT of degradant (min)
Acid	9.75	0.317	1.082	89.51	99.26	2.018, 3.917
Alkaline	15.15	0.198	0.501	83.83	98.98	1.422, 3.287
Neutral	5.8	0.227	0.691	93.31	99.11	2.584
Oxidative	12.25	0.358	0.828	87.74	99.99	3.496, 12.311
Photolytic	0	0.496	0.956	98.62	98.62	–
Thermal	0	0.101	0.472	99.01	99.01	–

*Average of three determinations

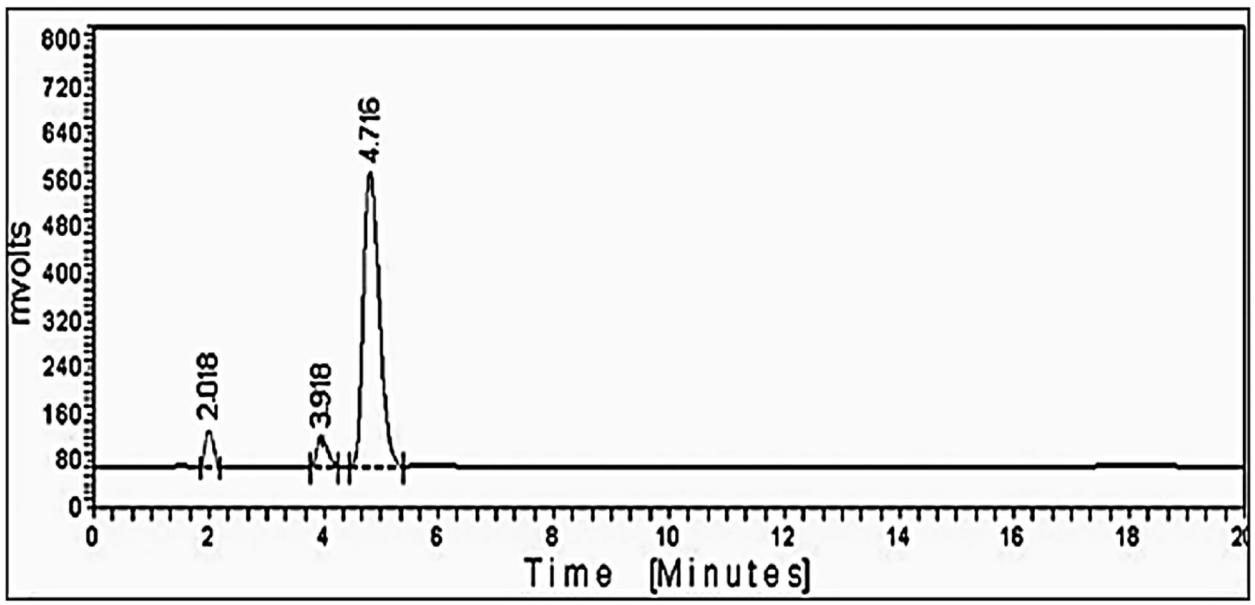

Fig. 5 Chromatogram of acid-treated DOX

that produced the requisite precision and accuracy are summarized in Table 2.

Precision

To control the precision data, interday and intraday examinations were checked out. The six replicate samples for the intraday experiment were injected at concentration during the same day. To analyze interday precision, samples of different concentrations over different days were injected to check out any variation of results. The precision result was found to be strong enough producing in an acceptable range. The data of the study are presented in Tables 3 and 4, respectively.

Accuracy

The accuracy study was conducted by taking a powdered sample of marketed preparation and further addition of known three different levels of samples with low, medium, and high (80%, 100%, 120%) with three replicates of each sample. The standard statistical analysis of the extracted sample for recovery is evaluated and presented in Tables 5 and 6.

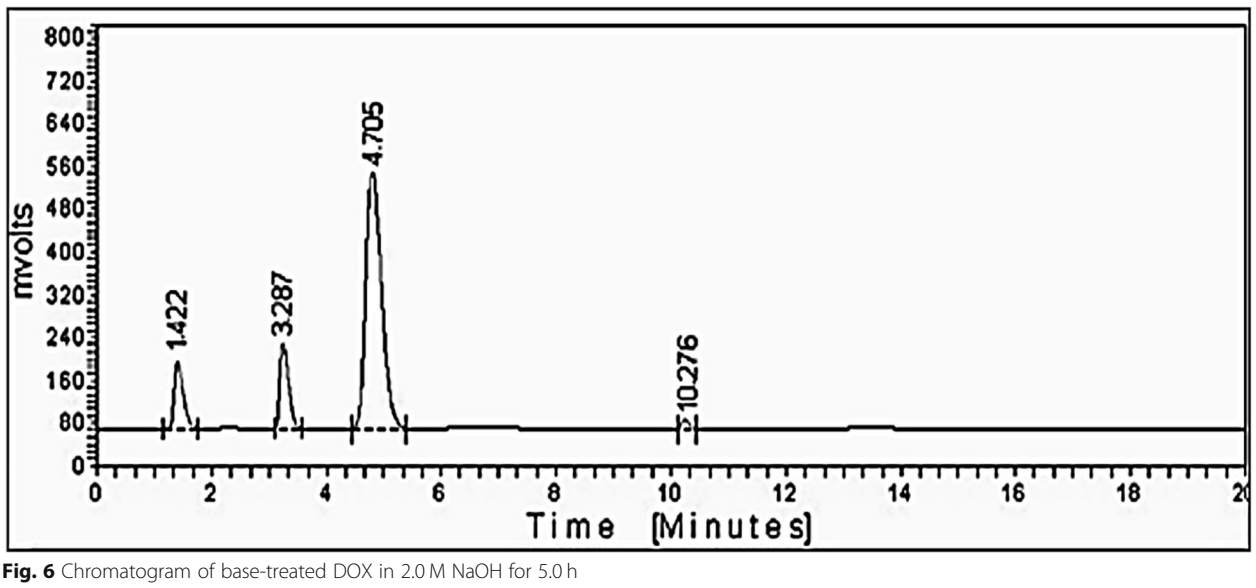

Fig. 6 Chromatogram of base-treated DOX in 2.0 M NaOH for 5.0 h

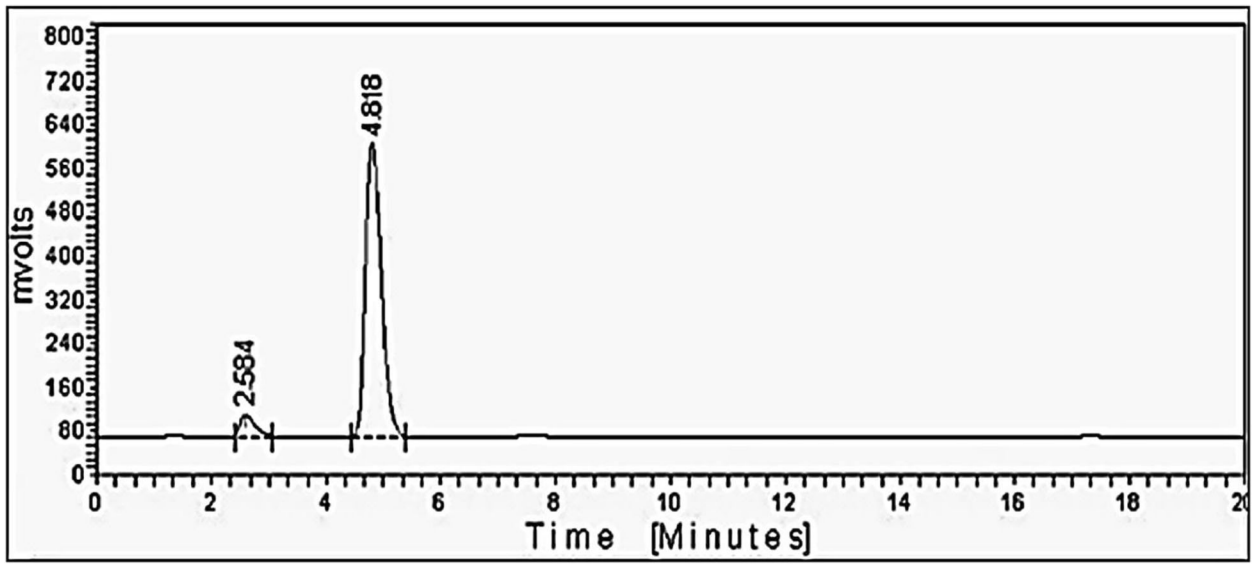

Fig. 7 Chromatogram of water hydrolysis of DOX after 10 h

Specificity

The specificity of the developed method reveals the high degree of specificity for DOX. The result of the degradation product of the marketed formulation showed that no interference of another element leads to a well-resolved peak with resolution factor > 2 in all samples. The PDA study demonstrates a separate identity of all peaks represented by purity index and purity threshold for pure peaks. The established mass balance study ensures all degradants were adequately detected.

Robustness

A robustness experiment was conducted to check the reliability of the developed method by the addition of small designed changes in chromatographic conditions such as a change in detection wavelength, flow rate, pH, and organic phase composition. The % RSD for triplicate injected sample was found to be < 2 indicating a robust method. Table 7 presents the results of the robustness of the recommended RP-HPLC method.

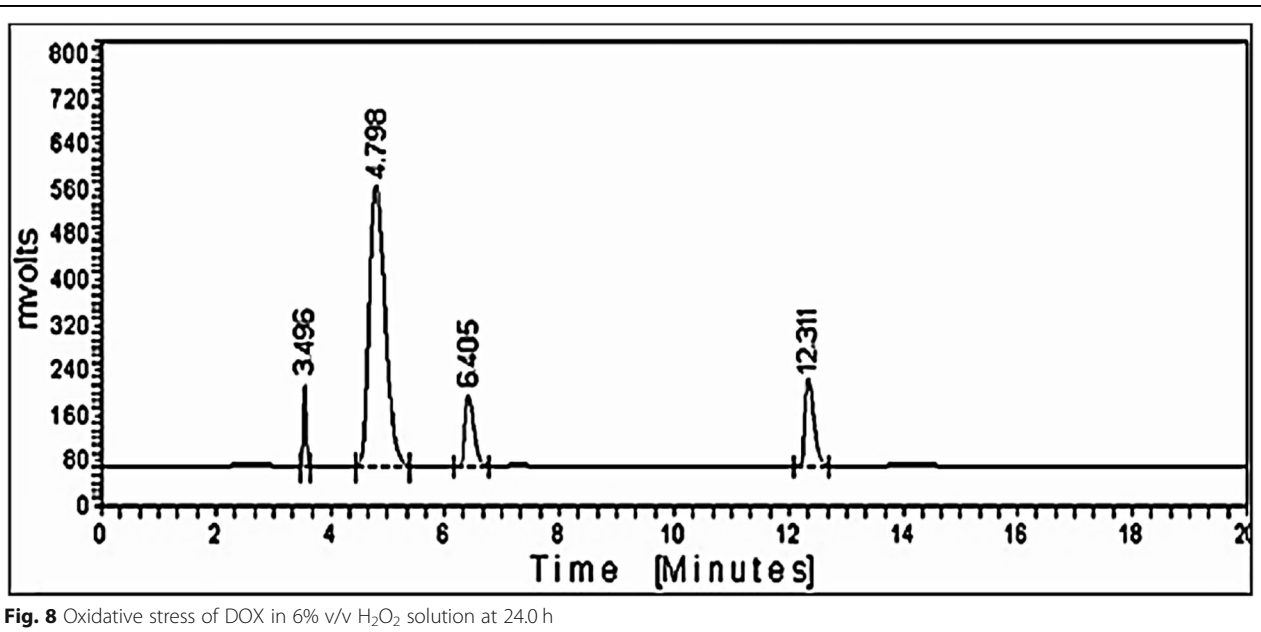

Fig. 8 Oxidative stress of DOX in 6% v/v H_2O_2 solution at 24.0 h

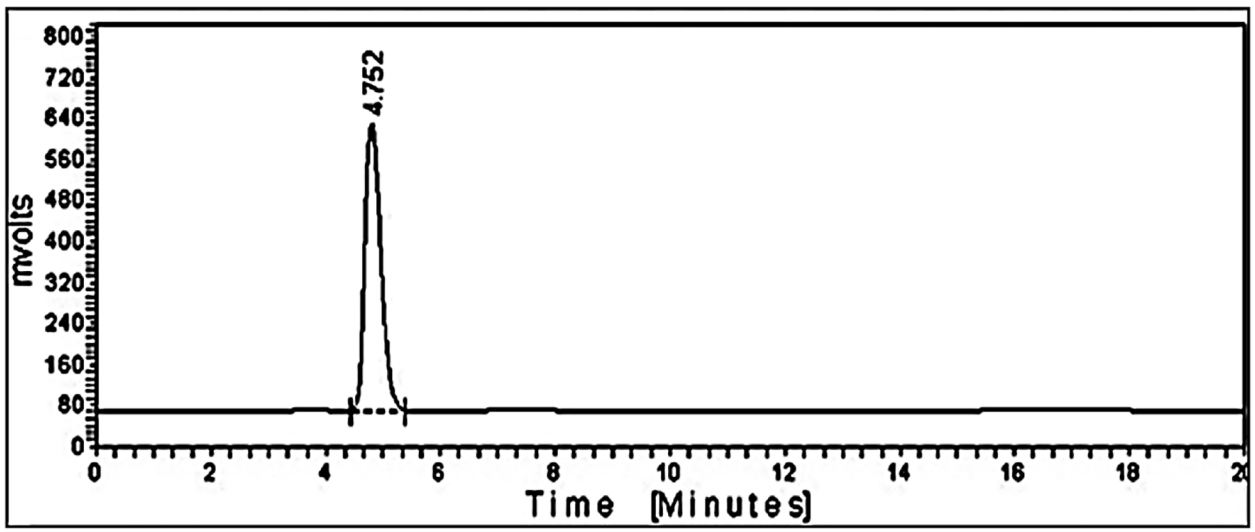

Fig. 9 Chromatogram of photolytic degradation of DOX after 72 h of study

Analysis of marketed formulation

The suggested method was applied to quantitative evaluation of restavit (25 mg) in pharmaceutical dosage form using the established RP-HPLC method. The comparative determination was performed between the peak area of the standard and sample. The data obtained after the assay of the marketed formulation was found to be in good agreement with the previous label claim (Table 8). The results of the statistical analysis are represented in Table 9.

Solution stability

The short-term solution stability of DOX (100 μg/ml) was evaluated under (25 ± 2°C) storage condition. The sample solution was assayed at 30 min and 1, 4, 8, and 24 h. The stability data showed the developed formulation remains stable after 24 h. To minimize errors during the experiment, all tests were performed in a period of 24 h and the results are summarized in Table 10.

Degradation behavior of DOX

The degradation experiment was conducted to analyze the stability and specificity of the recommended method. For the study, the sample was exposed to 2 M HCl at 70°C and found to be highly labile and exhibit degradation within 8 h followed by major degradation retention time (RT) 2.0 and 3.9 min. The exposure of 2 M NaOH for 5 h at70°C exhibits major degradation at relative RT

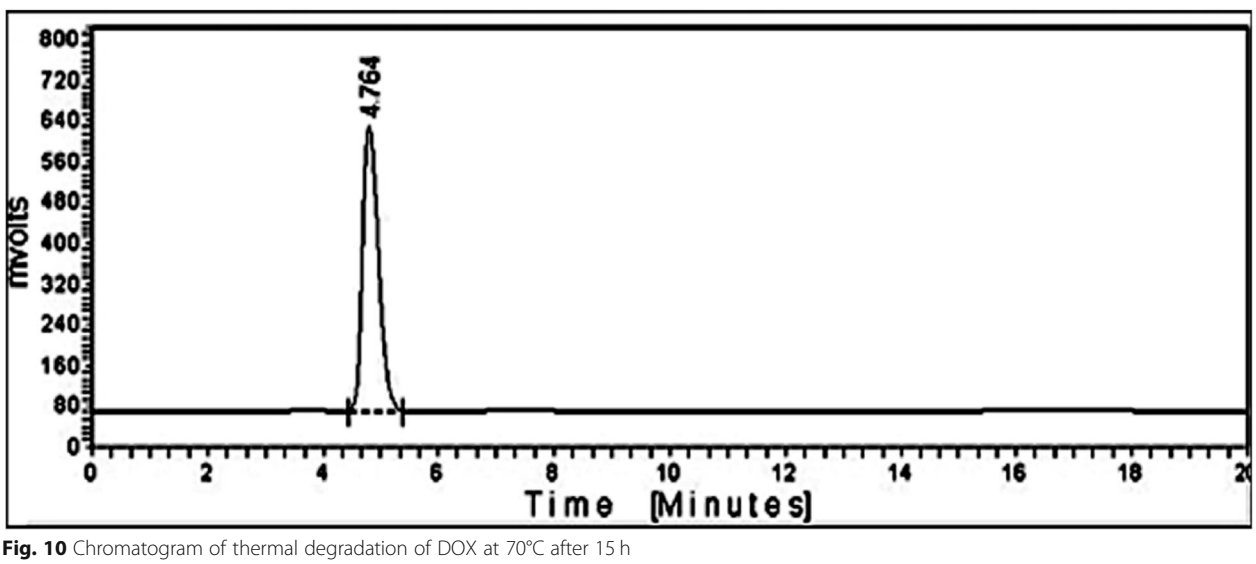

Fig. 10 Chromatogram of thermal degradation of DOX at 70°C after 15 h

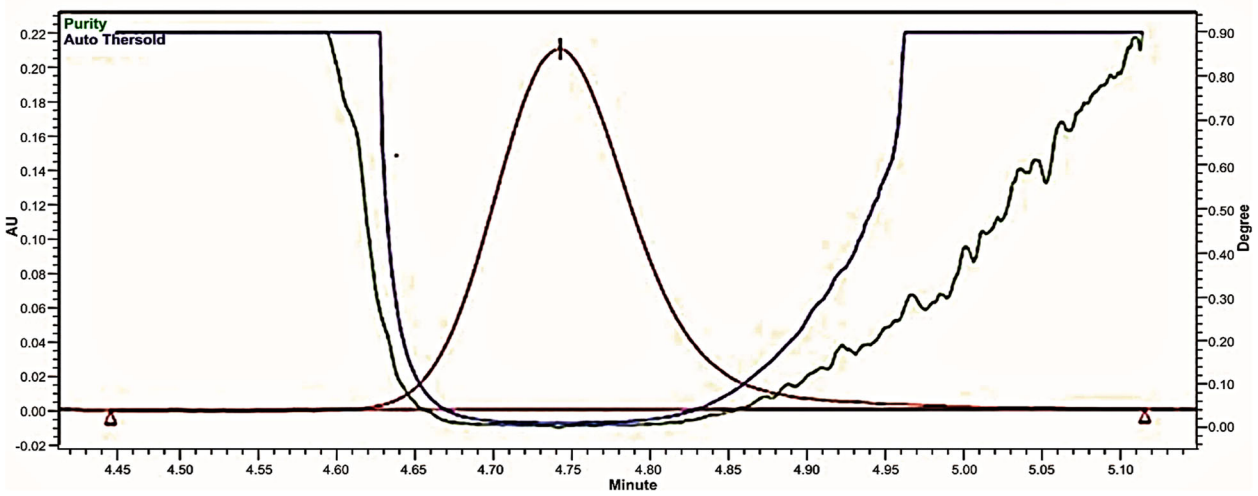

Fig. 11 Peak purity spectra

1.4 and 3.29 min. Natural degradation maintained reflux condition at 70°C for 10 h with no interference observed and RT appeared at 2.5 min. Similarly, oxidative degradation (exposure to 6% H_2O_2) for 24 h appeared separation at 3.4 and 12.3 min manifest sensitivity for oxidative stress condition. Thermal degradation (70°C for 48 h) and photolytic degradation (exposure UV for 48 h at 70°C) representing the absence of coeluent of degradant exhibit peak purity value within specificity suitable limit. The obtained densitogram is represented in Figs. 5, 6, 7, 8, 9, and 10. All stressed samples were mixed together and peak purity spectra were obtained with the help of a PDA detector in order to determine the potential of the developed HPLC method to resolve degradants from drug peak. The obtained peak purity spectra are represented in Fig. 11 whereas the mass balance and peak purity study of DOX are summarized in Table 11.

Identification of alkaline degradation product of DOX

The division of primary amine from DOX (Fig. 12) architecture was culminated due to the presence of 2 M NaOH at 70°C for 5 h. The IR and mass spectra were used to carry out structural elucidation of alkaline degradation of the product. The IR spectrum demonstrates to C-H stretch, N-H stretch,

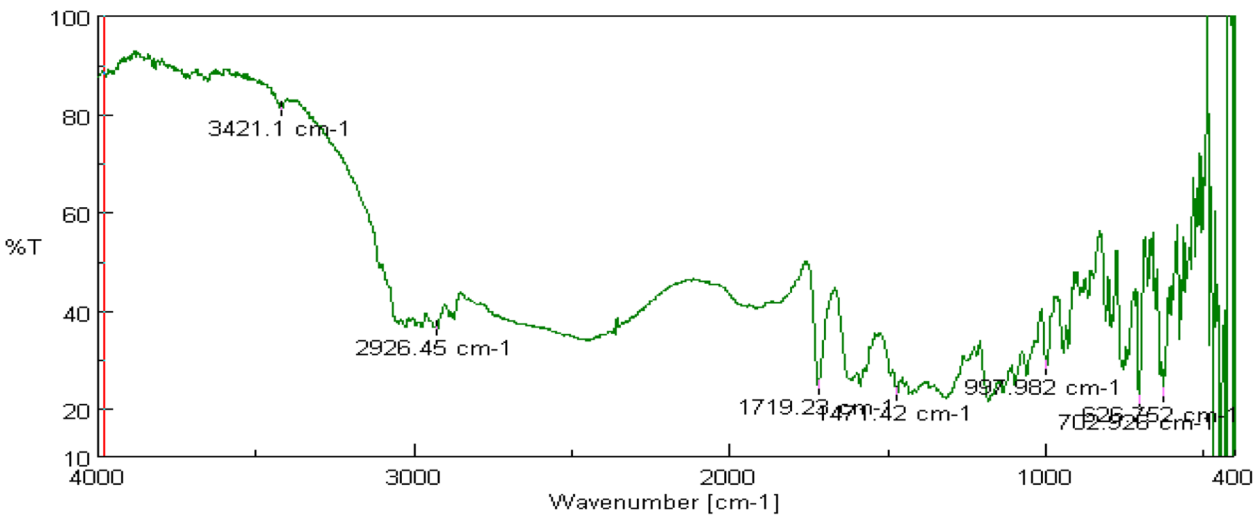

Fig. 12 FT-IR spectra of the standard DOX

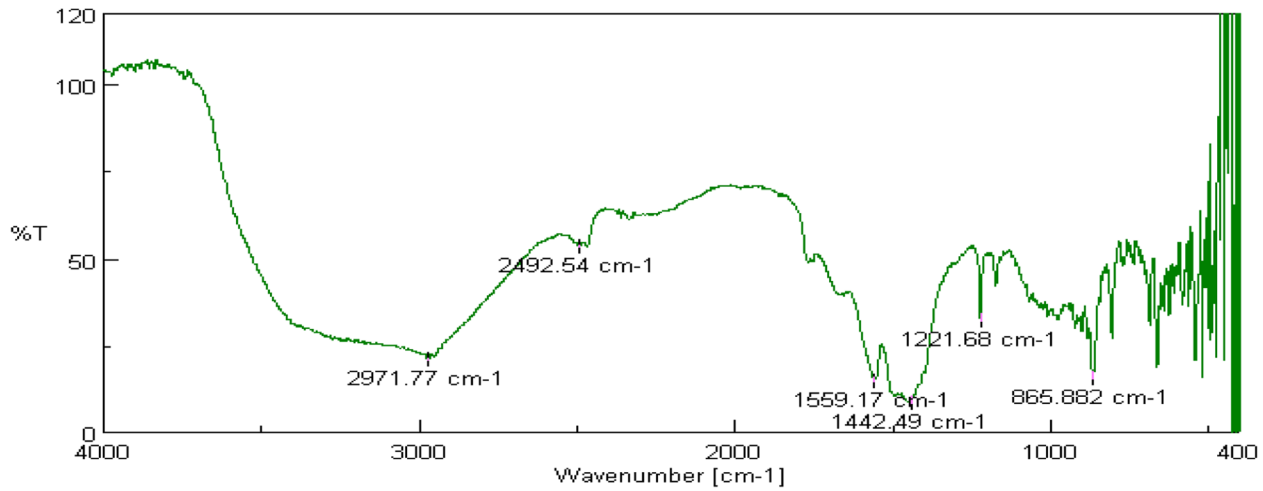

Fig. 13 FT-IR spectra of the alkaline degradant

and R-O-R ascribed to DOX (Fig. 13) characteristics. The pure DOX mass (Fig. 14) spectra express molecular ion peak at *m*/*z* 271.25 illustrating the molecular weight of doxylamine. Spectral data of degradant (Fig. 15) was characterized by breakage of primary amine from the structure of DOX indicating the structure of 2[α−methyl−α (2-pyridyl) benzyloxy] ethyl group which was confirmed by the appearance of molecular ion peak at *m*/*z* 226.29.

Discussion

The developed RP-HPLC [30] method of DOX plays a significant role and is capable of identifying separation between the active ingredient and degradant product. Analyte condition was set at a 262-nm wavelength with chromatographic separation using Kromasil C_{18} (4.6 × 250 mm, 5 μm). The good separate symmetrical peak was obtained with a combination of the mobile phase. The LOD and LOQ are the least quantity of analyte and least amount of analyte required to find out the method's accuracy and precision which were found to be 0.96 and 3.28 μg/ml, respectively [31]. The recommended experiment was also designed for commercial formulation and found to be 99.86% which indicates acceptable agreement with the label claim. The intraday repeatability of the precision study was carried out and it meets the requirement of < 2% RSD, whereas interday experiments were executed at different days and expected to recover results with intermediate agreement. The results of the study were found to be not less than 2%. The small variation in % RSD was received after slight adjustment in parameters like change in flow rate, change in mobile phase composition, change in detection wavelength, and pH of the mobile phase. The data obtained from the experiment proved the robustness of the method. The stress degradation study was carried out and the retrieved result showed well-resolved degradation peaks of DOX.

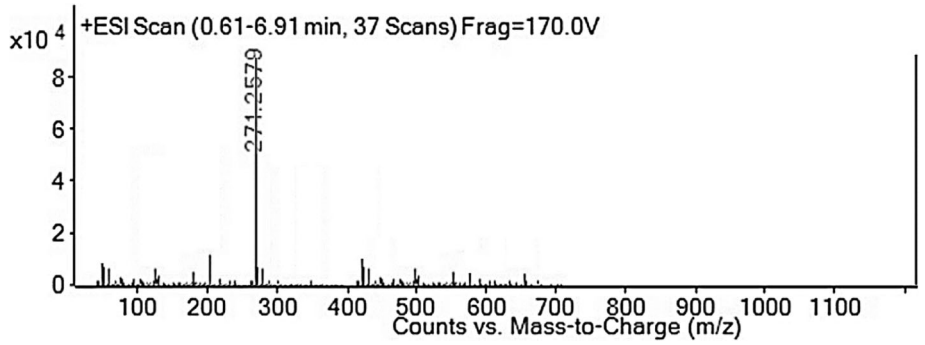

Fig. 14 Mass spectra of the standard DOX

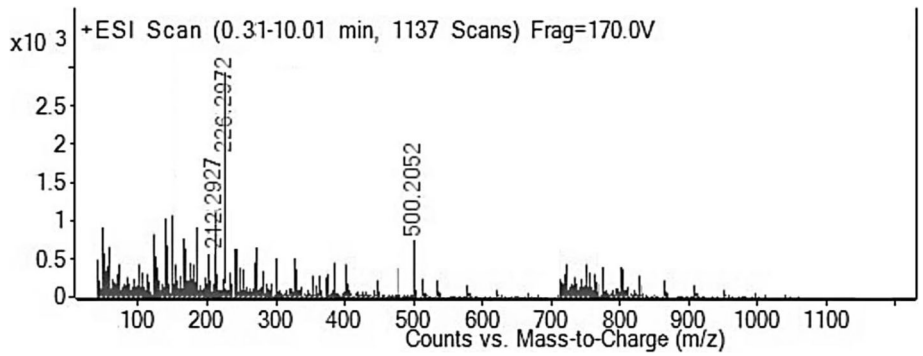

Fig. 15 Mass spectra of the alkaline degradant of DOX

Conclusion

In conclusion, simple, optimized, validated RP-HPLC methods for quantification of DOX as per the ICH guidelines were estimated. The further developed method was found to be useful in the separation of degradant in pharmaceutical dosage form and in different stress conditions. The recommended method confirmed linearity and is cost-effective, selective, reproducible, and more benefited for routine analysis of DOX. In our present knowledge, no method was explored in the literature for DOX determination. The various degradation product of DOX at different stress conditions was separated without any errors, clarifying and accomplishing the objective of the current investigation.

Abbreviations

LOD: Limit of detection; LOQ: Limit of quantitation; DOX: Doxylamine succinate; % RSD: Relative standard deviation; HPLC: High-performance liquid chromatography; NMT: Not more than; NLT: Not less than; min: Minutes; MT: More than; ICH: International Conference on Harmonization; Rs: Resolution; S. D.: Standard deviation

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Authors' contributions

All authors have read and approved the final manuscript; MTH conducted the literature survey, designed the work, performed the experiment, and developed and validated the new RP-HPLC method and statistical analysis with a graph. SHL contributed to the research design and edited the manuscript, monitored all the experiments, reviewed the data, and supervised activities.

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Availability of data and materials

All data and material are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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