RESEARCH ARTICLE

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Chemical Sciences Journal, Vol. 2012: CSJ-67

Development and Validation of Stability Indicating Analytical Method for Doxazosin Mesylate and its Application to Kinetic Studies

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Accepted: Aug 17, 2012; Published: Sep 08, 2012

Abstract
A stability-indicating LC assay method was developed for the quantitative determination of Doxazosin mesylate (DXM) in pharmaceutical dosage form in the presence of its degradation products and kinetic determinations were evaluated in acidic and alkaline degradation conditions. Chromatographic separation was achieved by use of LiChroCART-Lichrosphere100 RP-18 column (250 × 4.0 mm, 5 μm). DXM degraded in acidic, alkaline and hydrogen peroxide conditions, while it was more stable in thermal and photolytic conditions. The described method was linear over a range of 1.0-300 μg mL^{-1} for determination of DXM (r= 0.9992). The acid degradation of DXM in 1M HCl solution showed zero-order kinetics with rate constant 0.45 mole $liter^{-1}$ $minutes^{-1}$, while the alkaline degradation with 1M NaOH demonstrated first-order kinetics with rate constant k = 0.0044 min^{-1}. The half-life ($t_{0.5}$) and shelf life ($t_{0.9}$) values were also determined for both the kinetic studies. F-test and t-test at 95% confidence level were used to check the intermediate precision data obtained under different experimental setups; the calculated value was found to be less than critical value. The developed method was found to be simple, specific, robust, linear, precise, and accurate for the determination of DXM in pharmaceutical formulations.

Keywords: Doxazosin mesylate; stability-indicating assay; stress degradation study; kinetic degradation.

1. Introduction

Doxazosin mesylate, l-(4-Amino-6,7-dimethoxy-2-quinazolinyl)-4-[(2,3-dihydro-l,4-benzodioxin-2-yl)carbonyl] piperazine (DXM), is a selective α_1-adrenergic blocker used in the management of hypertension [1-3]. Several methods have been reported for the determination of DXM in plasma [4-10] and pharmaceutical formulations [11-14], including HPLC, LC-MS, UV spectrophotometry [15] and HPTLC [16]. However, literature has demonstrated stability-indicating LC methods by Bebawy *et al.* for the determination of DXM and celecoxib [17]. Nevertheless, this study assayed the DXM and celecoxib using UV and HPTLC methods. Thus, the aim of our study was to develop and validate a simple stability-indicating LC method, which allowed the determination of DXM in pharmaceutical dosage form, and also to determine the kinetics of degradation describing the concentration changes of DXM in acid and alkaline degradations.

2. Methods

2.1. Materials

DXM reference standard (91.1% of DXM free base) was obtained from Dr Reddy Labs (Hyderabad, India). DXM tablets (Duracard® 2 mg) were purchased from the market. HPLC grade methanol was purchased from Rankem, India, and high pure water was prepared by using Millipore Milli Q plus purification system. Hydrogen peroxide was purchased from Qualigens Fine Chemicals, India and sodium hydroxide was purchased from Merck Ltd.

2.2. Apparatus and chromatographic conditions

Quantitative HPLC was performed on Shimadzu HPLC with LC 10 AT VP series pumps besides SPD 10 A VP UV-Visible detector. The chromatographic separations were performed using LiChroCART-Lichrosphere100, C18, RP column (250mm × 4mm × 5μm) maintained at ambient temperature, eluted with mobile phase at a flow rate of 1ml/min for 10 min. The output signal was monitored and integrated using Shimadzu Class-VP version 6.12 SP1 software. The mobile phase consisted of methanol-water (60:40 % v/v). Measurements were made with injection volume 20μl and ultraviolet (UV) detection at 247 nm.

2.3. Preparation of standard and sample solutions

Stock standard solution of DXM (100 μg mL^{-1}) was prepared in methanol. An aliquot of this solution was diluted in the mobile phase to obtain the final concentration of 10 μg mL^{-1} of DXM. To prepare a sample solution, twenty weighed tablets of Duracard® (2 mg of DXM) were ground and an amount of powder equivalent to 10 mg of active compound was diluted with methanol and then sonicated for 20 min. The sample solution was filtered and the appropriate aliquot was diluted in the mobile phase to obtain a final solution containing 10 μg mL^{-1} of DXM.

2.4. Method validation

The validation procedure for the analysis of DXM by LC method followed the International Conference on Harmonization (ICH) guideline and United States Pharmacopoeia [18-20]. The performance parameters evaluated in this method were specificity, robustness, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy.

2.4.1. Specificity and forced degradation studies

The specificity of the LC method was evaluated to ensure that there was no interference from the excipients contained in pharmaceutical product or from products resulting from forced degradation. A stability-indicating method is the one that accurately quantifies the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities [21, 22]. This greatly contributes to the possibility of improving drug safety [23, 24].

Solutions containing 1 mg mL^{-1} of the drug were prepared in methanol for the stress degradation studies. An appropriate aliquot was transferred into a volumetric flask and the volume was completed with 0.5 M NaOH, 1 M NaOH, 1.5 M NaOH, 0.5 M HCl, 1 M HCl, 1.5 M HCl, 3% H_2O_2, 5% H_2O_2 and 10% H_2O_2 to give a final concentration of 100 μg mL^{-1} of DXM. These solutions were subjected to heating at 70 ºC. The hydrolytic study was carried out in 0.5 M NaOH for 1 h, 1 M NaOH for 45min, 1.5 M NaOH for 30min, 0.5 M HCl for 1 h, 1 M HCl for 45min, 1.5 M HCl for 30min. The oxidative reaction was performed with 3% H_2O_2 for 1 h, 5% H_2O_2 for 30min and 10% H_2O_2 for 15min at room temperature. For LC analyses, 1.0 mL aliquots of the above solutions were transferred to 10 mL volumetric flasks, neutralized as needed, and each sample diluted to the mark with mobile phase.

The stress degradation study under UV radiation was performed by exposing the DXM solution in methanol (1 mg mL^{-1}) for 30min and 1h at room temperature, resulting in an overall illumination of ≥210Wh/m^2 with UV radiation at 320–400 nm in a photostability chamber (1.0 x 0.17 x 0.17 m) with mirrors and equipped with UV lamps and UV cuvettes, were used as a container for samples. Samples submitted to identical conditions, but protected from light, were used as a control. Similarly the samples for thermal studies are exposed to a controlled-temperature oven at 80°C for 30min and 1 h.

2.4.2. Robustness

Chromatographic parameters (peak retention time, theoretical plates, tailing factor, retention factor, and repeatability) were evaluated using both samples and reference substance solutions (10 μg mL^{-1}) changing wavelength (245 and 249 nm), column temperature (23 and 27 ºC), flow rate (0.8 and 1.2 mL min^{-1}) and methanol concentration (58 and 62%).

2.4.3. Linearity

Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curves (n=3) were linear over the concentration range of 1–300μg/ml. Peak areas of DXM were plotted against their respective concentrations and linear regression analysis was performed on the resultant curve.

2.4.4. LOD and LOQ

LOD and LOQ were determined by reducing the concentration of a standard solution until the DXM peak response was approximately three or ten times greater than the noise, respectively.

2.4.5. Precision

The precision of the proposed method was evaluated by carrying out six independent (50μg/ml) assays of test sample. RSD (%) of six assay values obtained was calculated. Intermediate precision was carried out by analyzing the samples by a different analyst on another instrument.

2.4.6. Accuracy

The accuracy of the method was determined through the recovery test of the samples, using known amounts of DXM reference standard. For LC method, aliquots of 0.8, 1.0 and 1.2 mL of a DXM standard solution (100 μg mL^{-1}) were added to three sample solutions containing a fixed amount of DXM (100 μg) in mobile phase, respectively. Therefore, this recovery study was performed at a final concentration solution of 80, 100 and 120 μg mL^{-1} DXM. All solutions were prepared in triplicate and analyzed.

2.4.7. System suitability test

System suitability tests were performed to ensure that the LC system and procedure are capable of providing quality data based on USP 31 requirements [16]. The system suitability parameters include DXM retention time, tailing factor and number of theoretical plates, as well as the peak area relative-standard deviation (RSD, n= 6) of reference standard.

2.4.8. Kinetic determinations

The kinetics of acid degradation of DXM was evaluated in 1M HCl at 70 ºC for different times. Tablets of Duracard® were ground and an amount of powder equivalent to 10 mg of DXM was dissolved in 10 mL methanol. The solution was filtered and an aliquot was transferred into 25 mL volumetric flask and diluted with 1M HCl to yield 100 μg mL^{-1} DXM. This solution was placed at 70 ºC and evaluated for time intervals of 0, 9, 18, 27, 36 and 45 min. Three samples were analyzed for each time interval. After the required time, aliquots of 1 mL were transferred to a 10 mL volumetric flask then neutralized with 1 mL 1M NaOH. This solution was diluted with mobile phase to 10 μg mL^{-1} for the LC determinations. Similarly, kinetics of alkaline degradation was also studied on 100 μg mL^{-1} DXM solution with 1M NaOH placed at 70ºC and neutralized with 1M HCl, followed by evaluation at different time intervals of 0, 9, 18, 27, 36 and 45 min.

The concentrations of the remaining DXM determined at the different time intervals in kinetics determination were used in the plots. The plots were (a) values of % remaining concentration against time (zero-order kinetics), (b) ln of remaining concentration versus time (first-order kinetics), (c) reciprocal of remaining concentration versus time (second-order kinetics), and (d) reciprocal of square of remaining concentration. The kinetic parameters such as order of degradation rate constant (*k*), half-life ($t_{0.5}$) and shelf life ($t_{0.9)}$ were also obtained.

3. Results and Discussion

3.1. Development of LC method

Regulatory agencies recommend the use of stability-indicating methods [20] for the analysis of stability samples [21]. Thus, stress studies are required in order to generate the stressed samples, method development and method validation [22]. In order to separate DXM and degradation products produced under stressed conditions, different mobile phases were used and adjusted to obtain a rapid and simple assay method with a reasonable run time, suitable retention time and the sharpness of the peak. Distinct proportions of organic solvent (methanol and acetonitrile) were evaluated and the methanol was chosen because it improved the retention time and symmetry of DXM peak. Buffer pH played a major role in separating all the degradation products of DXM. Thus, the mobile phase was established by mixing methanol and water (60:40, %v/v).

3.2. Method validation

3.2.1. Specificity and forced degradation studies

Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating methods, particularly when little information is available about potential degradation products. The ICH guideline titled “Stability Testing of New Drug Substances and Products” requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substances [15]. Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. The use of an ideal stability-indicating method quantifies the drug per se and also resolves its degradation products.

Stress degradation studies on DXM revealed the drug behavior, as summarized in chromatograms (Figures 1-5). Upon heating the drug in HCl and NaOH (0.5M, 1M, 1.5M) for different times (1h, 45min, 30min)

at 70°C, fall in the original drug peak areas were observed and no additional peak was observed in all the chromatograms (Figure 1 and 2). Photolytic degradation and thermal degradation resulted in slight decrease of the peak area and did not produce any detectable eluting degradation product (Figure 3 and 4). DXM was degraded in acidic and alkaline media proportional to the concentration of acidic and alkaline conditions and the peak area of DXM decreased substantially.

The DXM solution was exposed to chemical oxidation with 3% H_2O_2 for 1h and 5% H_2O_2 for 30min and 10% H_2O_2 for 15min, the oxidative hydrolysis with H_2O_2 exhibited a significant decrease of peak area and only one eluting peak was observed in all the chromatograms (Figure 5). The results of the stress conditions are presented in Table 1. Thus, our study demonstrated that DXM was degraded in acidic, alkaline media and in the presence of hydrogen peroxide too.

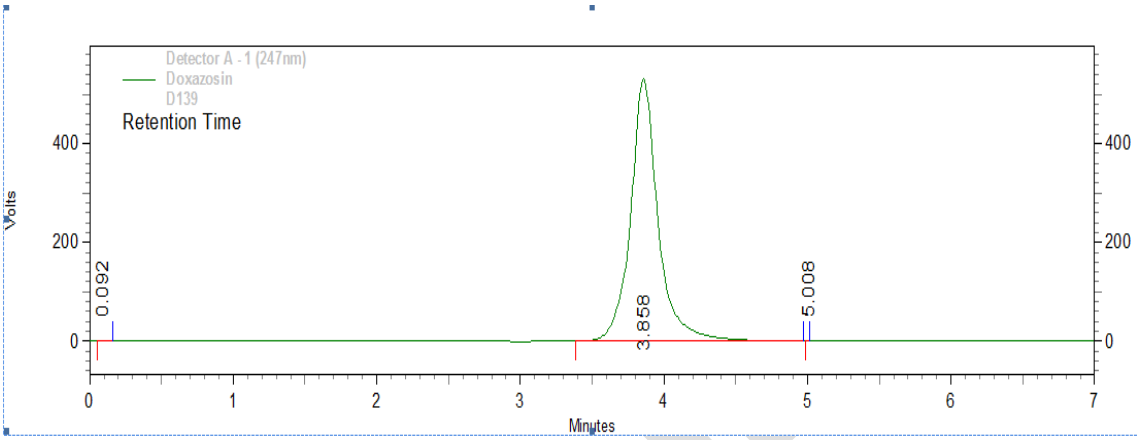

Figure 1: Representative chromatogram of DXM in acid degradation.

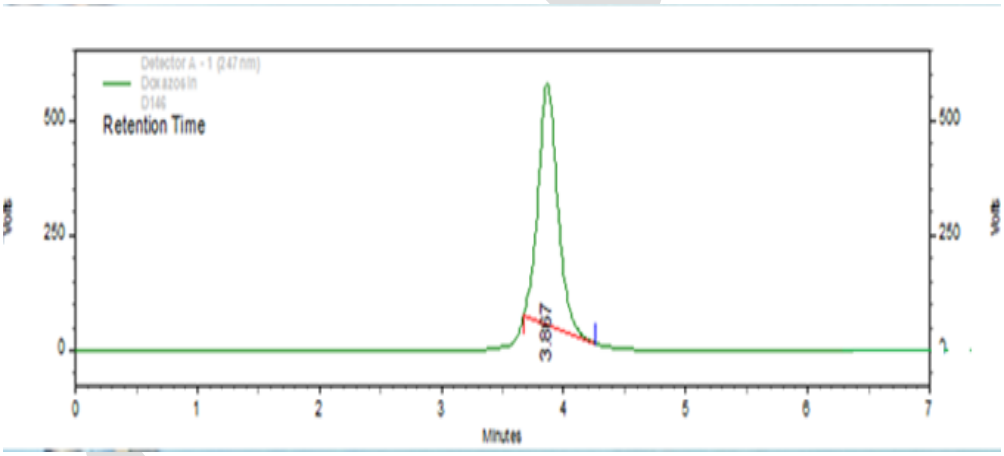

Figure 2: Representative chromatogram of DXM in alkaline degradaion.

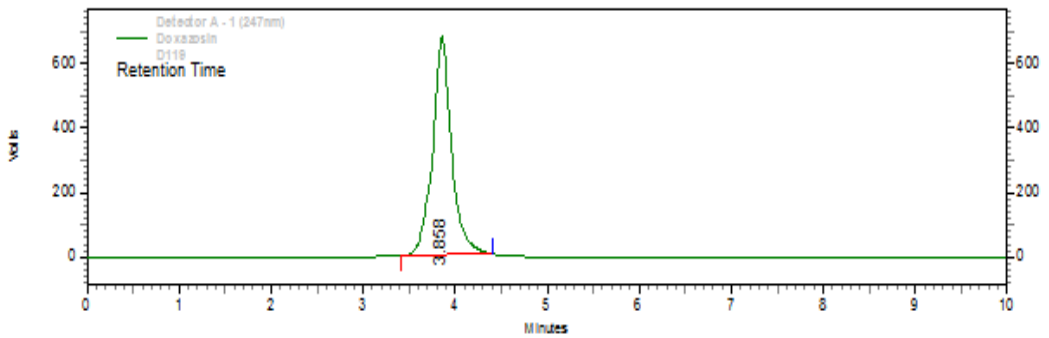

Figure 3: Representative chromatogram of DXM under UV light.

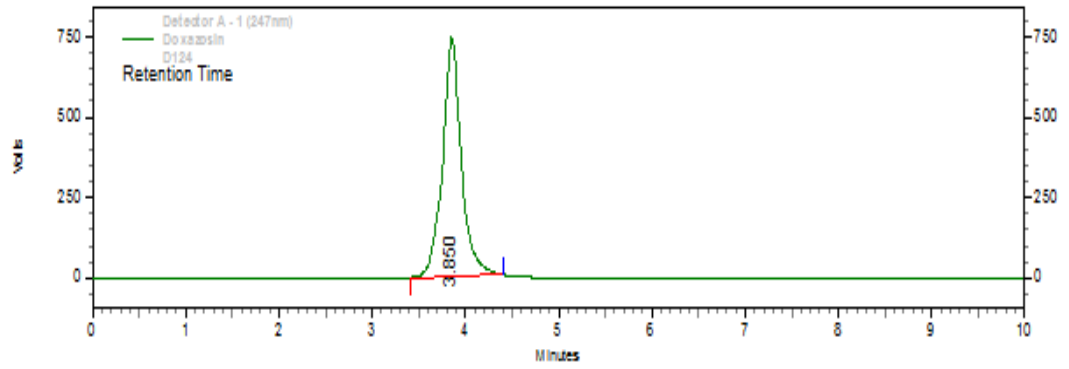

Figure 4: Representative chromatogram of DXM under thermal degradation.

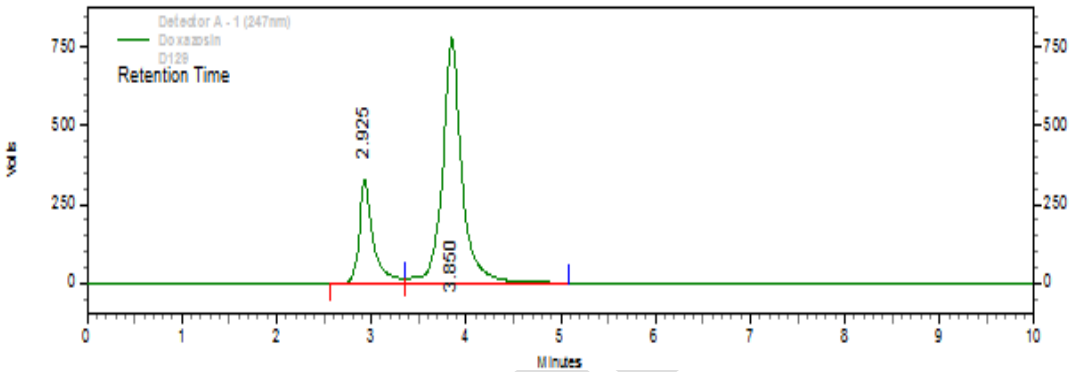

Figure 5: Representative chromatogram of DXM in peroxide degradation.

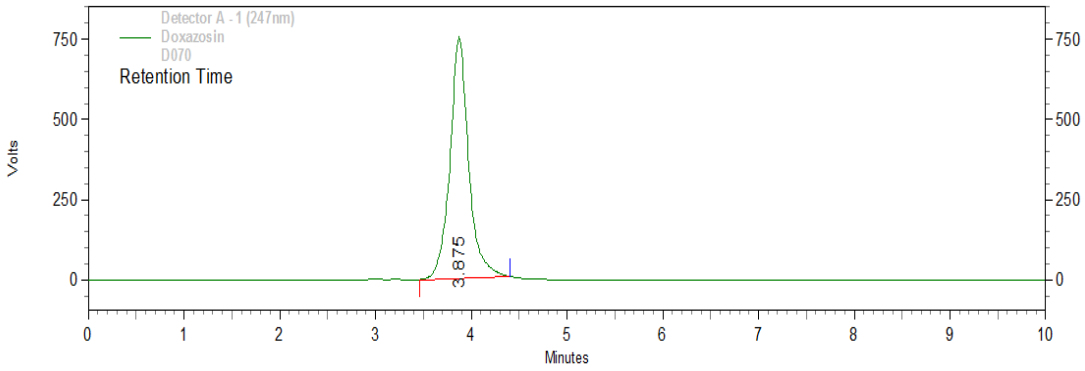

Figure 6: Representative chromatogram of DXM in reference standard.

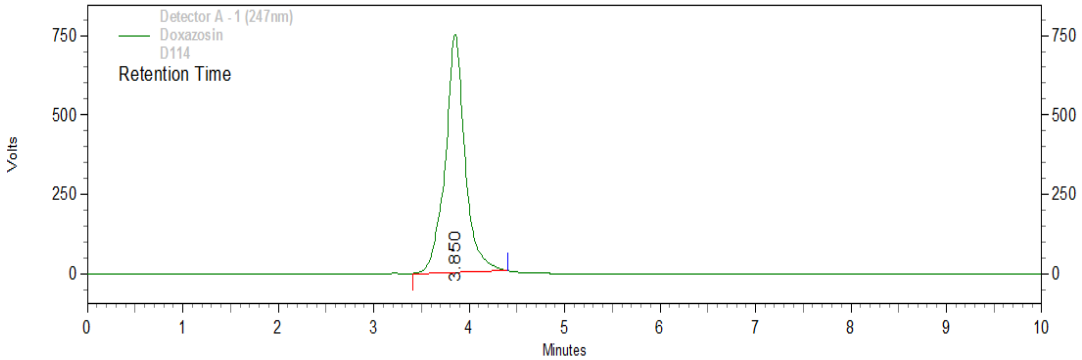

Figure 7: Representative chromatogram of DXM in pharmaceutical formulations.

The excipients of Duracard® tablets did not cause interference in the DXM analysis, which indicated the specificity of the method. Assay studies were carried out for stress samples against qualified reference standard. This demonstrated that it was pure in all cases indicating that no additional peaks were co-eluting with the DXM and evidencing the ability of the method to assess unequivocally the drug of interest in the presence of potential interferences. In order to consider an assay method specific, it should demonstrate that it could separate and quantify the drug from a physical mixture of the drug, degradation products and excipients.

Table 1: Summary of forced degradation results.

Stress condition	Time (Min)	% Assay of active substance	Remarks
Acid Hydrolysis (70°C)			No degradation product was formed in all the 3 conditions
0.5N HCl	60	87.31	
1N HCl	45	85.44	
1.5N HCl	30	88.79	
Base Hydrolysis (70°C)			No degradation product was formed in all the 3 conditions
0.5N HCl	60	81.23	
1N HCl	45	80.94	
1.5N HCl	30	82.78	
Oxidation (Room temp)			No degradation product was formed in all the 3 conditions
3% H_2O_2	60	79.73	
5% H_2O_2	30	82.80	
10% H_2O_2	15	76.26	
Thermal (80°C)	60	95.61	No degradation product was formed in both the conditions
	30	94.99	
Light (Photolytic degradation) at room temp	60	98.63	No degradation product was formed in both the conditions
	30	99.01	

3.2.2. Robustness

The robustness of the method was examined by small variations of critical parameters, and percent of DXM, retention time (R_t), number of theoretical plates (N) and tailing factor (T), were evaluated (Table 2).

Table 2: Robustness experiments of LC method for determination of DXM.

Chromatographic parameter	Condition	DXM (%)	R_t[a] DXM (min)	N[b]	T[c]
Wavelength (nm)	245	98.59	3.78	6601	1.17
	247	98.53	3.82	6615	1.17
Temperature (ºC)	23	97.99	3.74	6620	1.15
	27	97.47	3.62	6609	1.13
Flow rate (mL min^{-1})	0.8	99.88	3.850	6607	1.21
	1.2	100.05	3.859	6597	1.23
Methanol (%)	58	100.55	3.863	6613	1.14
	62	100.17	3.877	6605	1.01
	Normal[d]	100.02	3.856	6628	1.15

[a] R_t: retention time
[b] N: number of theoretical plates
[c] T: tailing factor
[d] Normal condition (mobile phase): LiChroCART-Lichrosphere100, C18, RP column (250 mm × 4 mm × 5 µm), methanol and water (60:40, % v/v), flow rate 1.0 mL min^{-1}, UV detection at 247 nm.

The robustness study has proved that in every employed condition, the chromatographic parameters agreed with established values and the assay data remained acceptable [15]. A tailing factor of 1 refers to a symmetric peak. The calculated values for the tailing factor for each chromatographic condition were in the acceptable range of $0.8 \leq T \leq 1.5$ [16]. The number of theoretical plates demonstrated the measure the column efficiency in different conditions. Flow rate (0.8 and 1.2 mL min^{-1}) and percent of methanol (58 and 62%) resulted in changes in the retention time in comparison with the proposed normal condition. However, no significant changes were observed regarding quantification of DXM.

3.2.3. Linearity

The standard curves for DXM were constructed and demonstrated to be linear in the concentration range of 1-300 μg mL^{-1}. The representative linear equation was $y = 102422x + 44166$, where x is the concentration (μg mL^{-1}) and y is the peak area. The correlation coefficient was $r = 0.9992$. Linearity data were validated by the analysis of variance (ANOVA), which demonstrated significant linear regression and no significant linearity deviation ($p < 0.05$).

3.2.4. LOD and LOQ

The limit of quantitation (LOQ) of the present method was found to be 1.2 μg/ml with a resultant %RSD of 0.63% (n = 5). The limit of detection (LOD) was found to be 0.3 μg/ml. The low values obtained were indicative of the high sensitivity of the method.

3.2.5. Precision

Precision values obtained for the determination of DXM in samples with their RSD are shown in Table 3. F-test and t-test were applied to the two sets of data at 95% confidence level, and no statistically significant difference was observed.

Table 3: Precision of DXM by proposed method.

Precision	DXM
	Mean assay (%) / %RSD
Set 1 (n=6) Set 2 (n=6)	99.8/0.929 99.6/0.871
	Calculated value / critical value
F-test t-test	0.993/3.368 0.528/2.106

3.2.6. Accuracy

Accuracy was evaluated by the simultaneous determination of the analyte in solutions prepared by the standard addition method. Three different concentrations of DXM standard were added to Duracard® tablet solution. The mean recovery was found to be 99.88% (Table 4) and this value showed that the method was accurate.

Table 4: Recovery of standard solution added to commercially available sample.

Amount added (μg ml^{-1})	Amount found (μg ml^{-1})	% Recovery[a] ± RSD	Mean % Recovery
80.0	79.67	99.58 ± 0.71	
100.0	99.88	99.88 ± 0.12	99.88
120.0	120.24	100.20 ± 0.33	

[a] Each value is a mean of three determinations.

3.2.7. System suitability test

The system suitability parameters evaluated, under the experimental conditions, showed a single peak of the drug around 3.8 min, tailing factor (T = 1.15) and number of theoretical plates (N = 6628), as well as the peak area relative-standard deviation (RSD = 0.8%, n = 6).

3.2.8. Assay

The validated method was applied to the determination of DXM in commercially available Duracard® 2mg tablets. Figure 6 and 7 illustrate two typical HPLC chromatograms obtained from DXM standard solution and from the assay of Duracard® tablets respectively. The results of the assay (n = 9) undertaken yielded 98.50% (%RSD = 1.5%) of label claim for DXM. The observed concentration of DXM was found to be 1.97±0.03µg/ml (mean±SD). The mean retention time of DXM was 3.8 min. The results of the assay indicate that the method is selective for the analysis of DXM without interference from the excipients used to formulate and produce these tablets.

3.2.9. Kinetic determinations

Most of the degradation reactions of pharmaceuticals occur at finite rates and are chemical in nature. These reactions are affected by conditions such as solvent, concentration of reactants, temperature, pH of the medium, radiation energy, and the presence of catalysts. The order of the reaction is described based on the reaction rate and on the concentration of the reactant. The degradation of most pharmaceuticals can be classified as zero order, first order or pseudo-first order [23]. Thus, kinetic studies of decomposition of drugs using stability testing techniques are essential for the quality control of such products. In our study, the kinetic investigation of acidic, alkaline and peroxide degradation was carried out.

The values of % remaining concentration, ln % remaining concentration, reciprocal of remaining concentration, and reciprocal square of concentration of the remaining drug versus time for kinetic determination under acidic and alkaline conditions are shown in Figures 6 and 7 respectively. Through the evaluation of the correlation coefficients, it could be concluded that the acidic degradation of DXM in 1M HCl solution showed zero-order kinetics (r = 0.9954) under the experimental conditions applied. The calculated zero-order acid degradation rate constant was k = 0.45 mole liter^{-1} minutes^{-1}, $t_{1/2}$ = 110.99 min and t_{90} = 22.19 min. Similarly, the correlation coefficient (r) observed under the experimental conditions applied in alkaline degradation study was 0.9941. The calculated first-order degradation rate constant was carried out and k = 0.0044min^{-1}, $t_{0.5}$ = 157.5 min and $t_{0.9}$ = 23.88min were obtained.

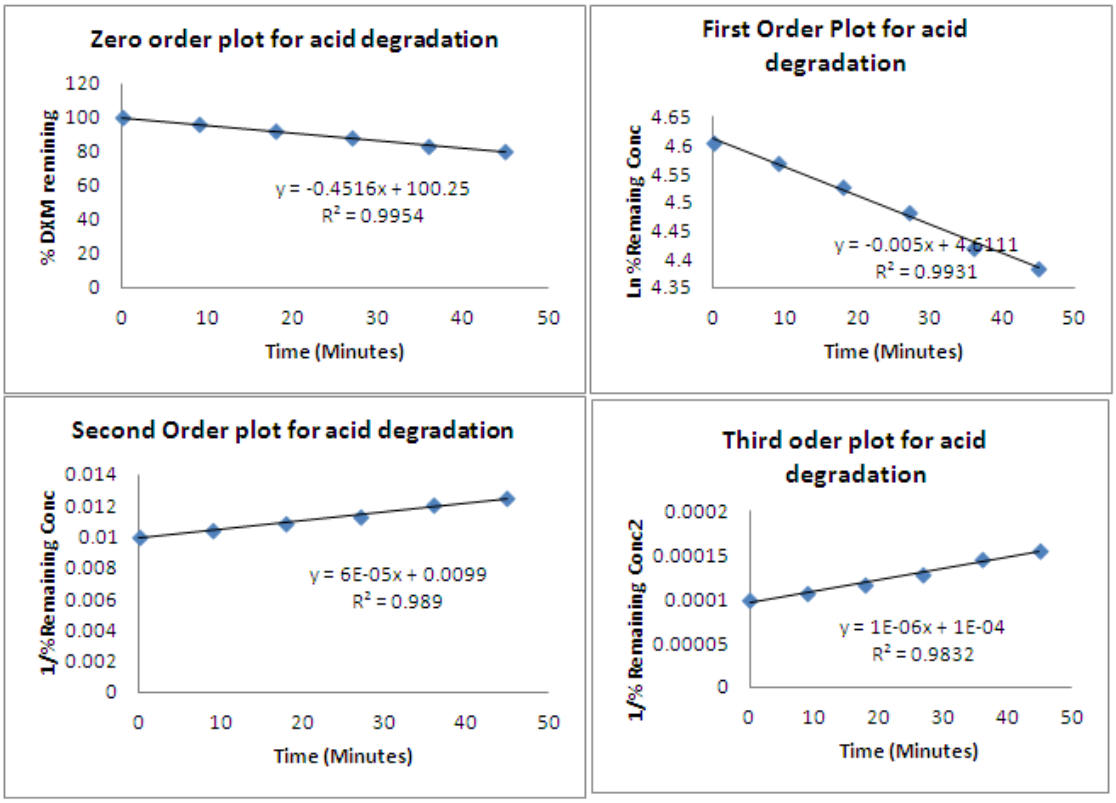

Figure 8: Acid degradation kinetic plots for DXM.

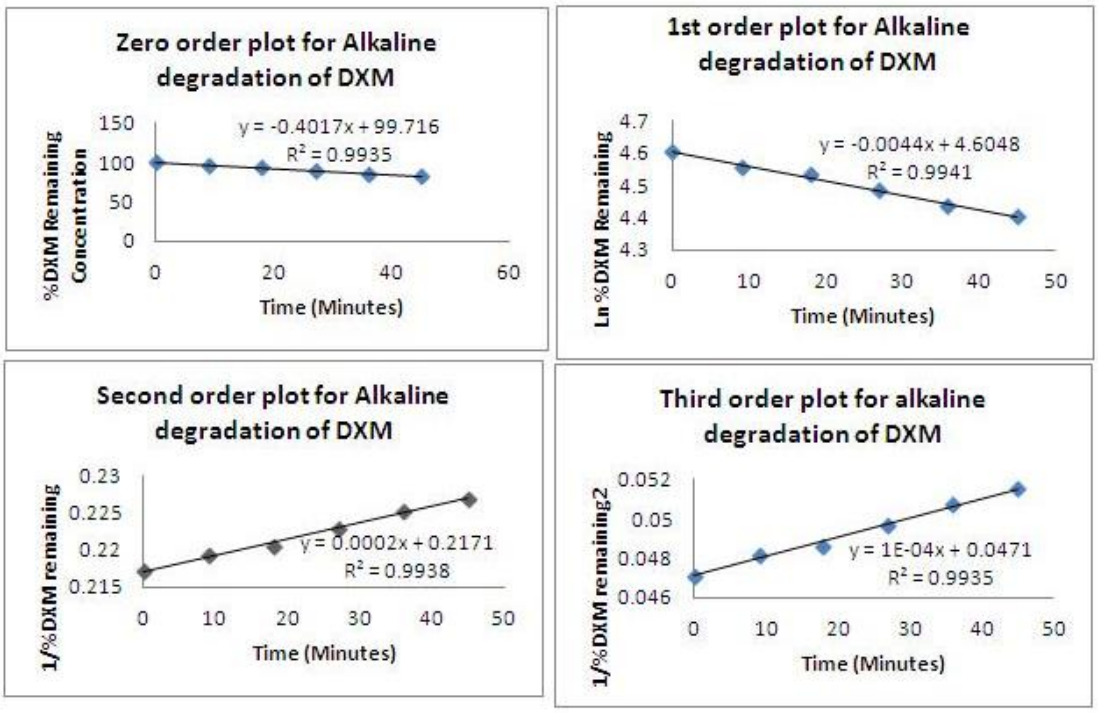

Figure 9: Alkaline degradation kinetic plots for DXM.

4. Conclusion

This paper has reported for the first time a simple analytical method for quantitative determination of DXM in pharmaceutical dosage form. The developed and validated LC method according to guidelines was simple, specific, linear, precise, accurate, and stability-indicating. DXM was rapidly degraded in acidic and alkaline medium and in the presence of hydrogen peroxide too, while it was more stable in UV radiation and thermal conditions. The acidic and alkaline degradations showed zero-order kinetics and first-order kinetics respectively. Kinetic parameters of degradation rate constant, $t_{1/2}$ and t_{90} could be predicted. The proposed LC method presented the ability to separate DXM from all its degradation products and therefore can be applied in stability testing of the commercially available DXM tablets.

Competing Interests

None declared.

Authors' Contributions

All authors contributed equally to this work.

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