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Article

Simultaneous RP-HPLC determination of sparfloxacin and dexamethasone in pharmaceutical formulations

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The present study describes the development and subsequent validation of simple and accurate stability indicating RP-HPLC method for the determination of sparfloxacin and dexamethasone in pharmaceutical formulations in the presence of their stress-induced degradation products. Both the drugs and their stress-induced degradation products were separated within 10 minutes using C8 column and mixture of methanol and 0.02 M phosphate buffer pH 3.0 (60:40 v/v, respectively) as mobile phase at 270 nm using diode array detector. Regression analysis showed linearity in the range of 15-105 µg/mL for sparfloxacin and 5-35 µg/mL for dexamethasone. All the analytes were adequately resolved with acceptable tailing. Peak purity of the two drugs was also greater than 0.9999, showing no co-elution peaks. The developed method was applied for simultaneous determination of sparfloxacin and dexamethasone in pharmaceutical formulations for stability studies.

Uniterms: RP-HPLC. Sparfloxacin. Dexamethasone. Degradation products. Stability studies.

O presente estudo descreve o desenvolvimento e a subsequente validação de indicador de estabilidade simples e acurada por RP-HPLC para a determinação de esparfloxacino e dexametasona em formulações farmacêuticas na presença de produtos de degradação induzidos por estresse. Tanto os fármacos quanto os produtos de degradação induzidos pelo estresse foram separados em 10 minutos, utilizando coluna C8 e mistura de methanol e tampão fosfato 0,02 M, pH 3,0 (60:40 v/v, respectivamente) como fase móvel e detector de arranjo de diodo a 270 nm, A análise de regressão mostrou linearidade na faixa de 15-105 µg/mL para esparfloxacino e 5-35 µg/mL para a dexametsona. Todos os analitos foram resolvidos adequadamente com tailing aceitável. O pico de pureza dos dois foi maior que 0.9999, não mostrando picos de co-eluição. O método desenvolvido foi aplicado para a determinação simultânea de esparfloxacino e dexametasona em formulações farmacêuticas e para estudos de estabilidade.

Unitermos: RP-HPLC. Esparfloxacino. Dexametasona. Produtos de degradação. Estudos de estabilidade.

INTRODUCTION

ICH and WHO recommended that analysis of pharmaceutical finished products during stability testing should be conducted by use of a validated stability-indicating method. In this study ICH and WHO recommendations were therefore kept in mind for the simultaneous determination of sparfloxacin and dexamethasone.

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Sparfloxacin is chemically designated as 5-amino-1-cyclopropyl-7-(*cis*-3,5-dimethyl-1-piperazinyl)-6,8-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid (Figure 1). It is a broad spectrum antibiotic and is used for the treatment of bacterial corneal infections, bacterial conjunctivitis and corneal ulcer to control the infections of the eye (Reynolds, 2009). Dexamethasone (Figure 1) chemically designated as (8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one is a corticosteroid, used

primarily for inflammatory ocular conditions (Reynolds, 2009). Individually sparfloxacin and dexamethasone have been analysed by various techniques. The techniques used for sparfloxacin include HPLC (Marona, Schapoval, 1999, 2001; Marona, Zuanazzi, Schapoval, 1999; Salgado *et al*., 2005; Al-Sayed, 1995), HPTLC (Mody *et al*., 1998), UV/VIS (Marona, Schapoval, 1999, 2001) and LC-MS (Noh *et al*., 2010). The analytical methods existed for dexamethasone include determination by HPLC (Iqbal *et al*., 2006; Huetos *et al*., 1999; Mallinson *et al*., 1995; Chen *et al*., 2008; Gallegos, Arroyo, 2002; Urban, Mainardes, Gremiao, 2009; Kwak, D'Amico, 1995), GC-MS (Mallinson *et al*., 1995) and thin layer chromatography (Huetos *et al*., 1999).

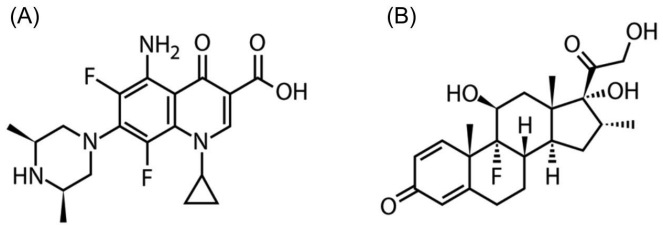

FIGURE 1 - Chemical structures of sparfloxacin (A) and dexamethasone (B).

The combination of sparfloxacin and dexamethasone has not been adopted by any official pharmacopoeia. An extensive review of the literature did not reveal any stability indicating HPLC method (with forced degradation studies) for simultaneous determination of both drugs. Therefore attempts were made to develop and validate simple and precise stability indicating RP-HPLC method for simultaneous determination of both drugs and their stress induced degradation products in pharmaceutical formulations. We are currently engaged in developing new HPLC methods for different classes of drugs in binary combination either in pharmaceutical formulations or in human plasma (Qutab *et al*., 2007a,b, 2009; Ashfaq *et al.,* 2007; Ashfaq, Khan, Asghar, 2008; Khan *et al*., 2008, 2010, 2012; Khan, Jilani, Ashfaq, 2010; Sharif *et al*., 2010.; Razzaq *et al*., 2012a,b,c,d). The present research work is a continuation of the work we have already reported.

MATERIAL AND METHODS

Chemicals and Reagents

Pure Reference standards (secondary standards) of sparfloxacin and dexamethasone with declared purity of 99.50 and 99.96% respectively were obtained from Schazoo Zaka Laboratories (Lahore, Pakistan). Spar-D eye drops (Biomedica International) claimed to contain 3 µg/mL of sparfloxacin and 1 µg/mL of dexamethasone (base) were used in this study. Methanol was of HPLC grade, whereas all other chemicals and reagents used in this study were of analytical reagent grade and were procured from M.S Traders Lahore, Pakistan (Fluka origin). Double distilled water was used for all the experiments. Filtration of the mobile phase was done using 0.45 µm nylon filters (Millipore, USA).

Equipment and chromatographic conditions

The HPLC system consisted of Shimadzu LC-20A system (Kyoto, Japan) equipped with model LC-20AT pump, SPD-M20A Diode array detector (set at 270 nm), and DGU-20A5 online degasser, and a Rheodyne injection valve with a 20 µL loop. Peak areas were integrated using a Shimadzu LC solution (version 1.227) software program. Experimental conditions were optimized on a Hypersil C 8 column (250 X 4.6 mm, 5 µm) at room temperature. Mobile phase was prepared by mixing 0.02 M phosphate buffer and methanol in ratio of 40:60 v/v, respectively. Phosphate buffer was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in 1000 mL of water. Its pH was adjusted to 3.0 with phosphoric acid after the addition of 1 mL of triethylamine. Mobile phase was flowed at 1.5 mL/min and all chromatographic experiments were performed at room temperature (25 ^{o}C ± 2 ^{o}C).

Preparation of standard solution

Standard stock solution of the two drugs was prepared by accurately weighing 37.5 mg sparfloxacin and 12.5 mg dexamethasone and then dissolved in few mL of methanol. The volume of this solution was then marked to 25 mL with 0.1 M sodium hydroxide and sonicated for 10 minutes. To prepare working standard solution, 1 mL of the above solution was diluted to 25 mL with mobile phase. The solution so obtained has concentration equal to 60 µg/mL sparfloxacin and 20 µg/mL dexamethasone. The solution was filtered using nylon filter before analysis.

Preparation of sample solution

1 mL commercial eye drop was diluted to 50 mL with mobile phase to obtain concentration equal to 60 µg/mL sparfloxacin and 20 µg/mL dexamethasone. The solution was filtered using nylon filter before analysis.

Linearity

Linear calibration plots of the proposed method were obtained over concentration ranges of 15-105 µg/mL (15, 30, 45, 60, 75, 90 and 105 µg/mL) for sparfloxacin and 5-35 µg/mL dexamethasone (5, 10, 15, 20, 25, 30 and 35 µg/mL). Triplicate analysis of each solution was carried out.

Accuracy

To demonstrate the accuracy of the proposed method, standard addition method was used. For this purpose, known quantities of sparfloxacin and dexamethasone were supplemented to the sample solution previously analysed. The results of this solution were compared with the true results. Three levels of solutions in the range of 50-150% of nominal analytical concentration (60 µg/mL sparfloxacin and 20 µg/mL dexamethasone) were prepared and analyzed.

Precision

Intra-day and inter-day precision was calculated to perform precision. Intra-day precision was measured by analyzing three different concentrations five times within the same day and inter-day precision were determined by evaluating the response of same solutions for three days. RSD of the peak area was then calculated.

Specificity (Stress Testing)

For specificity demonstration, acidic, basic, oxidative, thermal and photolytic stresses were applied as described by ICH in its guidelines. All stress studies were performed in 25 mL volumetric flask.

Acid Degradation Studies

Acid degradation study was performed in versatile environmental test chamber (Sanyo, Japan) at 40 °C/75% RH using 5 M HCl. For this purpose, 1 mL of the standard stock solution and 1 mL of 5 M HCl were taken in 25 mL volumetric flask and then kept in versatile environmental test chamber at 40 °C/75% RH. After 22 hours, those solutions were neutralized using 5 M sodium hydroxide and finally completed till the mark with mobile phase.

Base Degradation Studies

Base degradation study was performed in versatile environmental test chamber (Sanyo, Japan) at 40 °C/75% RH and at 22 °C/49% RH using 5 M NaOH. For this purpose, 1 mL of the standard stock solution and 1 mL of 5 M NaOH were taken in 25 mL volumetric flask and then kept in versatile environmental test chamber at 40 °C/75% RH for 16 h and at 22 °C/49% RH for forty five minutes. After the stated times, the solutions were neutralized using 5 M HCl and completed till the mark with mobile phase.

Oxidative Degradation Studies

Oxidative degradation study was performed in versatile environmental test chamber (Sanyo, Japan) at 40 °C/75% RH using 6% H_2O_2. For this purpose, 1 mL of the standard stock solution and 1 mL of 6% H_2O_2 were taken in 25 mL volumetric flasks and then kept in versatile environmental test chamber at 40 °C/75% RH for 22 h. After completion of the stress, the flask was completed till the mark with mobile phase.

Thermal Degradation Studies

Thermal degradation study was performed in versatile environmental test chamber (Sanyo, Japan) at 40 °C/75% RH. For this purpose, 1 mL of the standard stock solution in 25 mL volumetric flask was kept in versatile environmental test chamber at 40 °C/75% RH for 22 h. After completion of the stress, the 25 mL flask was completed till the mark with mobile phase.

Photolytic Degradation Studies

For photolytic degradation study, 1 mL of the standard stock solution in 25 mL volumetric flask was placed in the direct sunlight. After 1.25 h, the flask was completed with mobile phase till the mark.

Robustness

Premeditate variations were performed in the experimental conditions of the proposed method to assess the method robustness. For this, faint modifications were made in the operating conditions like mobile phase composition, flow rate and pH of buffer solution. The effect of these changes on chromatographic results was then measured.

RESULTS AND DISCUSSION

In this study, we developed a simple, sensitive and accurate RP-HPLC method for the separation of sparfloxacin and dexamethasone and their stress induced degradation products using C8 column. In order to obtain symmetrical peaks with better resolution, the chromatographic conditions i.e. pH of the buffer, concentration of organic modifier and flow rate of the

mobile phase were optimized. During first phase of this method development both methanol and acetonitrile were tried as organic content of the mobile phase along with phosphate buffer (pH 3.0; 20 mM) in order to get the peaks with better selectivity and good resolution. Use of acetonitrile resulted in broader peaks, whereas better peaks were obtained using methanol. Increase in temperature of the column oven from 30 to 50 °C did not improve the peak shapes of the analytes during acetonitrile use. So finally methanol was selected as organic portion of the mobile phase.

For selection of the buffer, phosphate buffer (pH 3.0; 0.02M) was taken because some researchers have used acid medium for the separation of corticosteroids and quinolones (Schil, Charles, 1994).

Hydrogen ion concentration has a definite control on the retention properties of substances so its influence on the separation efficiency of sparfloxacin and dexamethasone was studied. Different phosphate buffer solutions of the same strength but different pH (3, 4, 5, 6 and 7) were prepared to check the separation efficiency under different acidic conditions. The use of buffers of different pH did not result in change in retention times of the analytes but results in peaks with variable asymmetry. In all those conditions except at pH 3, there was considerable peak tailing for sparfloxacin, so pH 3 was finally selected for further studies.

The percentage of methanol in the mobile phase was varied from 40 to 80% in order to check the effect of organic modifier on the separation efficiency of the two analytes. Obviously, in RP-chromatography decreasing the polarity of the mobile phase results in faster elution so increasing concentration of methanol decreased the retention time of the analytes. Peaks with maximum selectivity and with all the chromatographic parameters within acceptable range were observed when using 60% methanol and 40% buffer within eight minutes as can be seen in Figure 2.

ICH guidelines were kept in practice for validation of the method (ICH,1996). Validation parameters performed include linearity, accuracy, precision, robustness, specificity, detection limit and quantitation limit.

Linear calibration plots for the proposed method were obtained in concentration ranges of 15-105 µg/mL (15, 30, 45, 60, 75, 90 and 105 µg/mL) for sparfloxacin and 5-35 µg/mL dexamethasone (5, 10, 15, 20, 25, 30 and 35 µg/mL). The linear regression equation for sparfloxacin was found to be Y= 51523 X + 7651 with correlation coefficient equal to 0.999 whereas for dexamethasone, it was Y= 81718 X + 2313 with correlation coefficient equal to 0.999.

The detection limit (DL) and quantitation limit (QL) were determined by making different solution with decreasing concentrations. DL was found to be 0.45 µg/mL and 0.15 µg/mL for sparfloxacin and dexamethasone, respectively (S/N ratio 3:1). QL was found to be 1.36 µg/mL and 0.45 µg/mL for sparfloxacin and dexamethasone, respectively (S/N ratio 10:1).

Accuracy of the method was performed by the standard addition technique. Three levels of solutions (50,

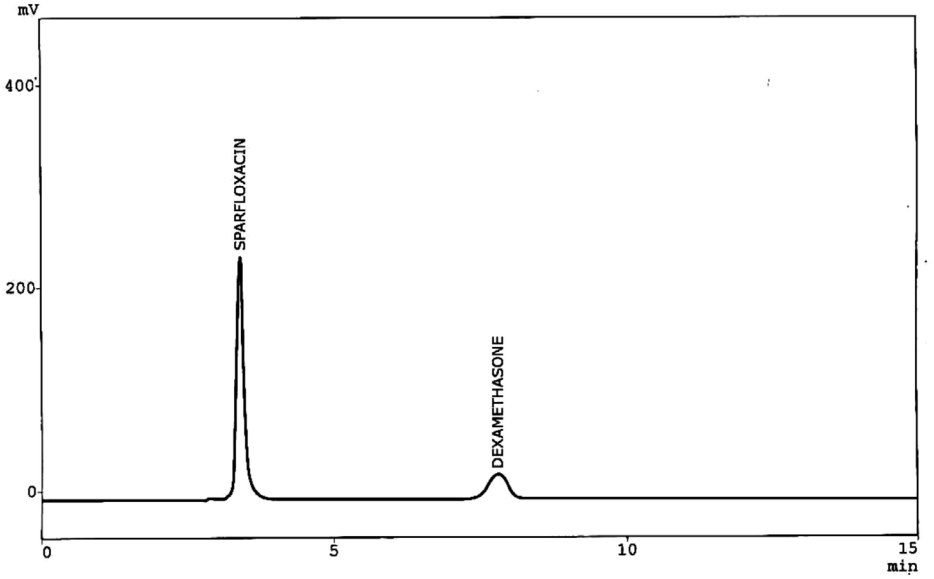

FIGURE 2 - Chromatogram of sparfloxacin (t_R = 3.415) and dexamethasone (t_R = 7.792) in pharmaceutical formulations. Chromatographic conditions: mobile phase methanol: 0.02 M phosphate buffer (60:40, v/v), pH 3.0, Column BDS Hypersil C8 (250 X 4.6, 5µm), flow rate 1.5 mL min^{-1}, injection volume 20 µL, wavelength 270 nm. Concentration: 60 µg/mL sparfloxacin and 20 µg/mL dexamethasone.

100 and 150%) of the nominal analytical concentrations were prepared. Percentage recoveries along with standard deviation and relative standard deviations for each analyte (n=5) are given in (Table I). Recovery studies showed the method to be highly accurate and suitable for intended use.

Intra-day and inter-day precision was calculated to perform precision. Intra-day precision was measured by analyzing three different concentrations five times within the same day and inter-day precision were determined by evaluating the response of same solutions for three days. Relative standard deviation (RSD %) of the peak area was calculated to represent precision. The results of intra-day and inter-day precision are presented in (Table II).

Robustness of the method was performed by slightly varying the chromatographic conditions. The results showed negligible effect on the chromatographic parameters by slight variations in chromatographic conditions (Table III and Table IV).

Specificity of the developed method was evaluated by applying different stress conditions (acid, base, oxidation, thermal and photolytic) to sparfloxacin and dexamethasone in combination form. The chromatograms under different stress conditions are shown in (Figure 3). The results of stress studies are given in (Table V).

All the stress conditions applied were enough to degrade both the drugs. Comparison of the two drugs showed that sparfloxacin is more stable as compared to dexamethasone. Under acidic conditions dexamethasone was degraded up to 97.3% and sparfloxacin was degraded up to 93.7. Under basic stress dexamethasone was

TABLE I - Accuracy of the Proposed HPLC Method

Drugs	Spiked concentration* (µg/mL)	Measured concentration* (µg/mL) ± SD; RSD (%)
Sparfloxacin	30.0	29.8 ± 0.7; 0.1
	60.0	60.1 ± 0.4; 0.7
	90.0	91.4 ± 0.5; 1.0
Dexamethasone	10.0	10.0 ± 0.2; 0.4
	20.0	20.1 ± 0.9; 0.5
	30.0	30.2 ± 0.7; 0.7

* = Average of 5 analysis

TABLE II - Intra-Day and Inter-Day Precision of the Proposed HPLC Method

Drugs	Actual Concentration (µg/mL)	Intra-day Precision Measured conc.; ± SD; RSD (%)	Inter-day precision Measured conc.; ± SD; RSD (%)
Sparfloxacin	30.0	30.2 ± 0.4; 1.1	29.6 ± 0.7; 1.2
	60.0	60.7 ± 0.8; 0.1	59.8 ± 0.8; 0.7
	90.0	90.1 ± 0.4; 1.5	89.8 ± 1.2; 1.1
Dexamethasone	10.0	10.0 ± 0.1; 0.4	10.0 ± 0.2; 0.3
	20.0	19.9 ± 0.2; 0.8	20.2 ± 0.5; 0.4
	30.0	29.9 ± 0.5; 1.0	30.0 ± 0.5; 0.1

TABLE III - Robustness study of sparfloxacin

Chromatographic Conditions	Assay %	t_R (min)	Theoretical plates	Tailing
Methanol:buffer (63:37)	100.1	3.221	4307	1.34
Methanol:buffer (60:40)	99.6	3.415	4358	1.34
Methanol:buffer (57:43)	101.5	3.515	4358	1.31
Flow rate (1.3 mL/min)	101.4	3.625	4398	1.32
Flow rate (1.5 mL/min)	100.8	3.415	4387	1.34
Flow rate (1.7 mL/min)	101.8	3.199	4325	1.34
Buffer (pH 2.8)	102.0	3.411	4358	1.32
Buffer (pH 3.0)	101.4	3.417	4322	1.34
Buffer (pH 3.2)	100.2	3.417	4378	1.34

TABLE IV - Robustness study of dexamethasone

Chromatographic Conditions	Assay %	t_R (min)	Theoretical plates	Tailing
Methanol:buffer (63:37)	100.4	7.214	2021	1.04
Methanol:buffer (60:40)	101.2	7.792	2074	1.04
Methanol:buffer (57:43)	102.0	8.311	2144	1.10
Flow rate (1.3 mL/min)	99.1	8.333	2254	1.10
Flow rate (1.5 mL/min)	99.5	7.792	2047	1.02
Flow rate (1.7 mL/min)	100.5	7.169	2178	1.03
Buffer (pH 2.8)	101.6	7.697	2298	1.02
Buffer (pH 3.0)	100.7	7.792	2258	1.04
Buffer (pH 3.2)	100.2	7.798	2241	1.10

TABLE V - Stress Testing Results of sparfloxacin and dexamethasone

Nature of stress	Storage Conditions	Time (h)	Amount of sparfloxacin	Amount of dexamethasone	Extent of decomposition
			Remaining±RSD (%)	Remaining±RSD (%)	
5M HCl	(40 °C/ 75 % RH)	22	6.3 ± 2.5 **(PPI=1.0000)**	2.7 ± 2.3 **(PPI=1.0000)**	**Substantial**
5M NaOH	(22 °C/ 49 % RH)	0.75	91.2 ± 1.4 **(PPI=1.0000)**	6.0 ± 2.1 **(PPI=1.0000)**	**Substantial**
	(40 °C/ 75 % RH)	16	8.04 ± 1.4 **(PPI=1.0000)**	4.0 ± 3.1 **(PPI=1.0000)**	**Substantial**
6% H_2O_2	(40 °C/ 75 % RH)	22	5.5 ± 1.0 **(PPI=1.0000)**	4.0 ± 1.1**(PPI=1.0000)**	**Substantial**
Thermal	(40 °C/ 75 % RH)	22	4.1 ± 1.3 **(PPI=1.0000)**	3.6 ± 1.5 **(PPI=1.0000)**	**Substantial**
Photolytic	Sunlight	1.25	98.7 ± 1.2 **(PPI=1.0000)**	3.0 ± 2.4 **(PPI=1.0000)**	**Substantial**

PPI= Peak Purity Index

degraded up to 96% and sparfloxacin was degraded up to 91.9%. Under oxidative stress dexamethasone was degraded up to 96% and sparfloxacin was degraded up to 94.5%. Under thermal stress dexamethasone was degraded up to 96.4% and sparfloxacin was degraded up to 95.9%. Under photolytic stress conditions dexamethasone was degraded up to 97.0% and sparfloxacin was found to be stable under photolytic stress. From these stress studies it was concluded that dexamethasone and sparfloxacin are not stable in basic, acidic, thermal, oxidative and photolytic stress conditions. The degradation pattern of the drugs is very similar under basic and photolytic stress conditions.

In addition to the percentage degradation of each drug, a number of degradation products (impurities) were produced under acidic, basic, thermal, oxidative and photolytic stress conditions (Figure 3). Application of the proposed method was checked by analyzing the sparfloxacin and dexamethasone in commercially available pharmaceutical products. The results are provided in (Table VI) which showed high percentage recoveries and low RSD (%) values for both analytes.

TABLE VI - Assay results of sparfloxacin and dexamethasone in commercial eye drops

Products Eye drops	Ingredient	Label value (mg/mL)	% Recovery* ±RSD (%)
Spar-D	Sparfloxacin	3	100.3 ± 0.3
	Dexamethasone	1	99.0 ± 0.7

* = Average of 10 determination

CONCLUSION

A simple, sensitive, isocratic and accurate reverse phase HPLC method is described for simultaneous determination of sparfloxacin and dexamethasone in pharmaceutical formulations. The proposed method was validated by testing its linearity, accuracy, precision, limits of detection and quantitation and specificity. The method was good enough to separate the peaks of active pharmaceutical ingredients (APIs) from the degradation products (produced during forced degradation studies). It is also clear from the chromatograms that both the active

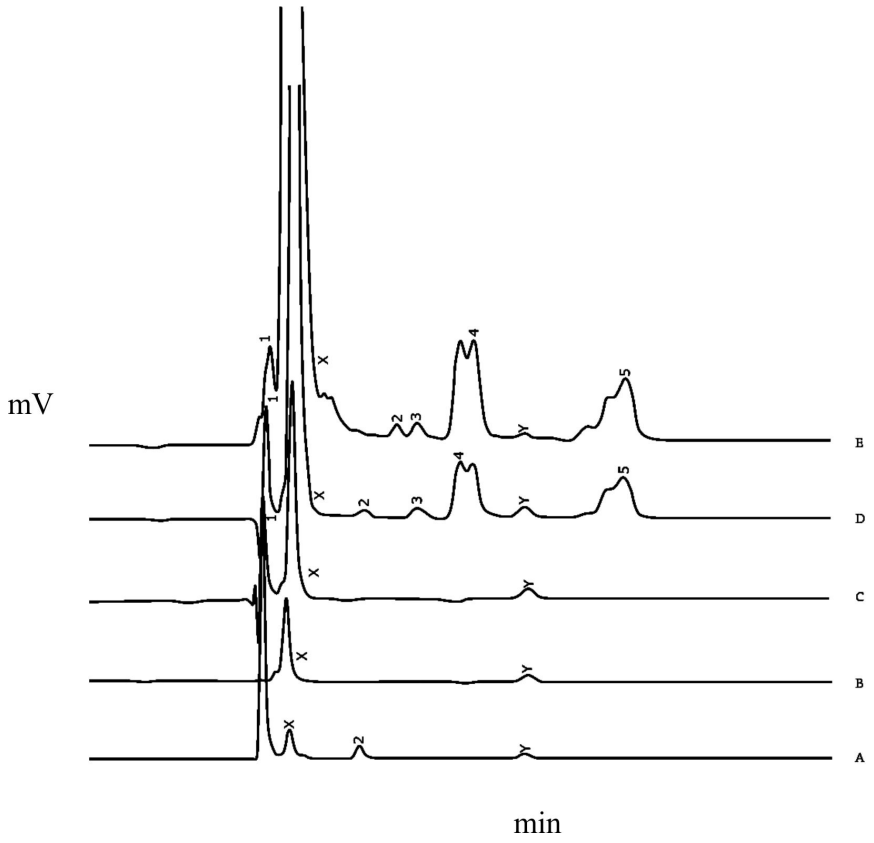

FIGURE - 3 A typical chromatogram of sparfloxacin (t_R = 3.425) and dexamethasone (t_R = 7.794) under acidic, basic, thermal, photolytic and oxidative stress conditions. Where (X) is sparfloxacin peak, (Y) dexamethasone peak, (1, 2, 3, 4, and 5) degradation/ impurities peaks, (A) chromatogram of oxidative stress, (B) chromatogram of thermal stress, (C) chromatogram of acidic stress, (D) chromatogram of basic stress and (E) chromatogram of photolytic stress. Chromatographic conditions: mobile phase methanol: 0.02 M phosphate buffer (60:40, v/v), pH 3.0, Column BDS Hypersil C8 (250 X 4.6, 5 µm), flow rate 1.5 mL min^{-1}, injection volume 20 µL, wavelength 270 nm.

ingredient peaks in all the stress conditions are free from any sort of degradation impurities. All these convince us to conclude that the method can be successfully used for any sort of stability and validation studies.

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