SPECTROFLUORIMETRIC METHOD FOR THE DETERMINATION OF AZELASTINE HYDROCHLORIDE IN BULK AND NASAL FORMULATIONS[**]

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A reproducible, sensitive, and cost-effective spectrofluorimetric method has been developed for the quantification of azelastine hydrochloride in its bulk and nasal formulations. The stability-indicating potential of the method was assessed by recovery studies in the forced degraded solutions of the drug. The method was validated in accordance with the ICH guidelines with respect to linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. Excellent linearity was noted in the concentration range 2.0–40.0 μg/ml with a correlation coefficient (R^2) of 0.9961. The limits of detection and quantitation for the proposed method were found to be 0.1598 μg/ml and 0.4845 μg/ml respectively. Excellent recovery of the drug was obtained from the proposed method in the nasal spray formulation of the drug (96.5, 0.66% RSD). The stability-indicating potential of the method was assessed from recovery studies of the drug from various forced-degraded samples spiked with known drug concentrations. The studies indicated a high rate of degradation in alkaline, oxidative, and photolytic stress degraded solutions.

Keywords: *azelastine, spectrofluorometric method, validation, analysis.*

СПЕКТРОФЛУОРИМЕТРИЧЕСКИЙ МЕТОД ОПРЕДЕЛЕНИЯ АЗЕЛАСТИНА ГИДРОХЛОРИДА В ОБЪЕМЕ И НАЗАЛЬНОЙ КОМПОЗИЦИИ

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УДК 543.42

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(Поступила 19 марта 2020)

Разработан воспроизводимый, чувствительный и экономичный спектрофлуориметрический метод количественного определения гидрохлорида азеластина в объеме и назальной композиции. Надежность метода оценена с помощью исследований восстановления лекарственного средства в искусственно разложенных растворах. В соответствии с ICH метод проверен в отношении линейности, точности, прецизионности, предела обнаружения (LOD), предела количественной оценки (LOQ) и воспроизводимости. Отличная линейность отмечена в диапазоне концентраций 2.0–40.0 мкг/мл с коэффициентом корреляции R^2 = 0.9961. Пределы обнаружения и количественного определения для предложенного метода 0.1598 и 0.4845 мкг/мл. Хорошее извлечение лекарственного средства достигнуто для случая назального спрея (RSD 96.5, 0.66%). Надежность метода оценена по результатам обнаружения лекарственного средства в различных подвергшихся искусственному разложению образцах, куда предварительно вносилось лекарственное средство с заданной концентрацией. Исследования показывают высокую скорость разложения азеластина в растворах, подвергшихся щелочным, окислительным и фотолитическим реакциям.

Ключевые слова: *азеластин, спектрофлуориметрический метод, валидация, анализ.*

[**] Full text is published in JAS V. 88, No. 3 (http://springer.com/journal/10812) and in electronic version of ZhPS V. 88, No. 3 (http://www.elibrary.ru/title_about.asp?id=7318; sales@elibrary.ru).

Introduction. Azelastine, 4-[(4-chlorophenyl)methyl]-2-(1-methylazepan-4-yl)phthalazin-1-one is a second-generation histamine H_1-receptor antagonist approved by the US-FDA for patients with seasonal allergic rhinitis (SAR) and vasomotor rhinitis [1, 2]. The fast and long-lasting effect of azelastine is attributed to its triple-pronged action, i.e., a combination of mast cell stabilization, anti-inflammatory, and anti-allergic effects [3, 4]. There are several reports on the detection of azelastine. The majority of these studies are based on chromatographic methods that include the estimation of azelastine and its related impurities by HPLC [5–9], HPTLC [10], UPLC, and capillary electrophoresis [11]. Quantitative determination of the drug was reported in bulk and pharmaceutical dosage forms (ophthalmic and nasal sprays) by densitometry TLC [12], potentiometry [13], and UV spectrophotometry [14–16]. Further, a couple of UPLC-based methods were reported for quantification of the drug in dietary supplements [17, 18]. Clinical determination of the drug in human plasma and metabolite profiling in a guinea pig were reported using HPLC [19, 20] and LC-ESI/MS/MS [21] methods. Blaschke et al. estimated the enantiomeric metabolites of azelastine in rats by LC–ionspray tandem mass spectrometry and electrokinetic capillary chromatography [22]. A stability-indicating mass spectrometry method was reported for the drug and its stress-degradation products [23]. Studies were also reported on the use of thermoanalytical techniques [24] and cyclic and differential pulse voltammetry [25] for the estimation of azelastine.

Spectrofluorimetric methods offer significant advantages over spectrophotometric methods in terms of higher sensitivity (up to 10^3 times) and selectivity attributed to the exclusive excitation/emission wavelength combination of a fluorophore. Moreover, spectrofluorimetric methods are more cost effective and convenient compared to HPLC ones. Hence, the present study was envisaged to develop and validate a simple and sensitive spectrofluorimetric method for the determination of azelastine in bulk and nasal spray formulations (EZICAS AZ®; Biodeal Pharmaceuticals). The method was validated with respect to various parameters outlined in the ICH guideline Q2(R1) [26]. Further, the drug was subjected to forced degradation studies under various ICH prescribed conditions. The degraded drug solutions were analyzed by the proposed method to assess the stability of the drug under various stressor conditions.

Experiment. *Chemicals and reagents.* Azelastine HCl (Batch number AH0030216, Material Code FP160184), was kindly gifted by Cipla Ltd., Mumbai (India). A nasal spray formulation containing azelastine HCl (0.14% w/v) (EZICAS AZ®), manufactured by Biodeal Pharmaceuticals Pvt. Ltd., Solan (Batch No. 1805080), was purchased from the local market. Absolute ethanol, sodium hydroxide, hydrochloric acid, and hydrogen peroxide (30%) were procured commercially from Merck India Pvt. Ltd., Mumbai. De-ionized water was prepared using a Milli-Q Plus purification system [Millipore, Bradford, USA], and the same water was used for the preparation of stressor solutions.

Instruments and apparatus. All the glassware, including volumetric flasks, pipettes, measuring cylinders, beakers, and test tubes, were of Class A grade, purchased from Borosil. Absorption and emission spectra were recorded using a Hitachi spectrofluorometer F 2500 with a scanning speed of 300 nm/min, 10 mm matched quartz cells, and a resolution of 2.5 nm. Hydrolytic samples were generated using a high-precision water bath shaker (Narang Scientific Works, New Delhi, India) capable of controlling the temperature within the range of ±1°C. Thermal stress testing samples were prepared in a hot air oven (Universal hot air oven, Ambala Cantt, India), equipped with digital temperature control (±2°C). Photodegradation was carried out in a photostability chamber (Dr. Honle, WTC Binder Photostability chamber, Binder Inc., USA, with a visible lux meter and a UV lux meter), with temperature and humidity control within the range of ±2°C and ±5% RH, respectively. The chamber was equipped with an illumination bank consisting of UV and fluorescent lamps as described in Option 2 in the ICH guideline Q1B [27]. The chamber was set at a temperature of 40°C and 75% RH.

Preparation of the drug stock solution. The standard stock solution for the spectrofluorometer (1000.0 μg/ml) was prepared daily by dissolving 10.0 mg of azelastine HCl in 10 ml of the solvent (absolute ethanol). This was diluted 1 in 10 to obtain a stock solution (100 μg/ml). The working standard solutions ranging from 2.0 to 40.0 μg/ml of azelastine were prepared by serial dilutions of the stock solution with absolute ethanol, and the test tubes were kept stoppered to avoid evaporation of the solvent.

Preparation of the calibration curves for azelastine HCl. The excitation and emission spectra for the working standard solutions of azelastine HCl (ranging from 2.0 to 40.0 μg/ml) were recorded over the wavelength ranges 200–400 and 400–800 nm, respectively. The maximum absorbance of the drug was observed at 291 nm (λ_{max}), which was selected as the excitation wavelength. The emission wavelength for the measurement of fluorescence intensity was selected as 359 nm. The optimized method was validated with respect to various parameters outlined in the ICH guideline Q2(R1).

Preparation of degradation solution. The drug concentration selected for stress studies was 1.0 mg/ml. Hydrolytic studies were carried out under acidic, basic, and neutral conditions by refluxing the drug in 1 N

HCl, 1 N NaOH, and water (triple distilled), respectively, at 80°C for 8 h. Photodegradation studies were carried out at 40°C by exposing a thin layer of the solid drug in a petri dish and also the drug solutions prepared in 0.1 N HCl, 0.1 N NaOH, and water, to a total dose of 1.2 million lux h of fluorescent and 200 Wh/m^2 of UV-A illumination in a photostability chamber, by placing them at about 9" from the light sources for 10 days. Oxidative studies were carried out at room temperature in 3% hydrogen peroxide (H_2O_2) for half an hour. Thermal degradation was carried out by exposing the drug (200 mg) in a petri dish, sealed with aluminum foil (to avoid photodegradation), to a temperature of 60^{o}C for 21 days. Subsequently, the petri dish was removed, cooled to room temperature, and its contents dissolved in acetonitrile (diluent).

The nasal spray formulation of azelastine was diluted 1 in 50 (0.2 ml drug formulation diluted up to 10 ml with absolute ethanol), and the aliquots were analyzed fluorometrically by the proposed method. All the dilutions were analyzed four times for the drug content. Further, the developed method was used for scrutinizing the drug content in stress-degraded samples of azelastine HCl. The solutions of all the stress-degraded samples were prepared by diluting 1 in 500 (0.02 ml of the stress-degraded samples diluted up to 10 ml) with absolute ethanol and were investigated three times. The stability indicating potential of the developed methods was evaluated by fortifying a mixture of the degraded solutions with three known concentrations of the drug. Equivalent volumes of the standard drug solutions (10, 20, and 40 μg/ml) were added to the degraded drug solutions (original drug concentration 10 μg/ml) so as to increase the drug concentration by nearly 50, 100, and 150% (final spiked drug concentrations, 5.0, 10.0, and 20.0 μg/ml, respectively).

Results and discussion. A sensitive spectrofluorimetric method of analysis for azelastine HCl has been developed and validated, and its stability indicating potential has been assessed. Azelastine contains a phthalazin-2-one moiety (Fig. 1), having a conjugated system of double bonds, in a planar stereochemical orientation, which should impart fluorochrome properties to the molecule. A systematic analysis of the fluorescence characteristics of the drug revealed that azelastine possesses good native fluorescence in an acidic medium and in ethanol; the pK_a value of azelastine is 8.88 [28]. Hence, the drug solubility was found to be higher in solutions with lower pH. The drug was found to be insoluble in basic medium. In the preliminary studies, drug solutions were prepared in various acidic buffers, and the corresponding fluorescence intensities were measured. The fluorescence intensities for the drug solution (5 μg/ml) in an ammonium acetate buffer (pH 3.8, 4.4, 5.8) were found to be low, ranging from 12.56 to 36.78 FU. Low-intensity fluorescence (around 25 FU) was also noted in phosphate buffer (pH 5.0). Maximum fluorescence (76.95 FU) was observed in ammonium acetate buffer (pH 5.1). The fluorescence intensity in ammonium formate buffer (pH 2.7, 3.5) ranged from 74.78 to 86.69 FU. Further, fluorometric testing was done in various solvents (water, ethanol, acetonitrile). Considering the UV absorption and solubility characteristics of the drug/solvent, ethanol was selected as the solvent, which returned good fluorescence intensities at the selected low drug concentrations. The stress-degraded samples were spiked with the pure drug in varying concentrations for analysis by the proposed method. The fluorescence intensity of the drug solutions/stress-degraded solutions was determined, taking 291 and 359 nm as the excitation and emission wavelengths, respectively, against the reagent blank. Figure 1 shows the excitation and emission spectra of azelastine HCl in absolute ethanol. The developed method was validated with respect to linearity and range, accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. The various validation parameters are summarized in Table 1.

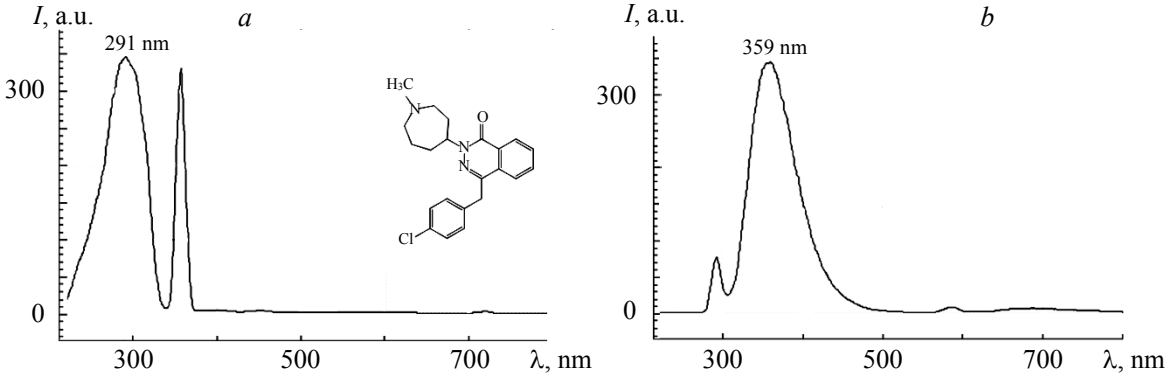

Fig. 1. (a) Excitation spectrum of azelastine HCl in absolute ethanol; (b) emission spectrum of azelastine HCl in absolute ethanol.

A strictly linear relation was observed between the fluorescence intensity and the concentration of azelastine in the concentration range 2.0–40.0 μg/ml. The fluorescence intensity in linearity studies for the proposed method is tabulated in Table 2. The calibration curve was described by the equation $y = 7.2997x + 110.26$ ($n = 3$, $R^2 = 0.9963$) (Fig. 2).

TABLE 1. Validation Parameters for the Proposed Method

Parameter		Azelastine HCl		
Accuracy	Concentration (μg/ml) ± S.D.; %RSD			
	Concentration of the drug, μg/ml	Concentration of the added standard, μg/ml	Calculated	%Recovery
	10.0	5.0 (50%)	14.24±12.71; 5.93%	94.9
	10.0	10.0 (100%)	18.50±2.54; 1.03%	92.5
	10.0	15.0 (150%)	24.32±1.28; 0.44%	97.3
Precision	Calculated concentration (μg/ml) ± S.D.; %RSD			
	Concentration taken, μg/ml	Intra-day ($n = 6$)	Inter-day ($n = 3$)	
	4.0	3.37±0.153; 0.11%	3.30±0.316; 0.24%	
	10.0	11.03±0.173; 0.09%	11.12±0.788; 0.41%	
	30.0	29.94±3.95; 1.20%	30.35±0.157; 0.05%	
Linearity	Range, μg/ml	Slope	Intercept	R^2
	2.0–40.0	7.2997	110.26	0.9963
LOD	0.1599			
LOQ	0.4845			

TABLE 2. Fluorescence Intensities in the Linearity Studies for the Proposed Method

Drug concentration, μg/ml	Fluorescence intensities		
	Set I	Set II	Set III
2.0	119.3	118.9	121.1
4.0	134.9	135.6	133.9
6.0	150.6	153.6	148.9
10.0	189.9	191.6	190.5
15.0	221.3	220.1	223.1
20.0	264.9	268.6	262.5
30.0	330.9	331.5	330.5
40.0	394.5	399.5	391.5
Slope	7.2974	7.3758	7.2268
Intercept	109.94	110.32	110.53
R^2	0.9964	0.9961	0.9958

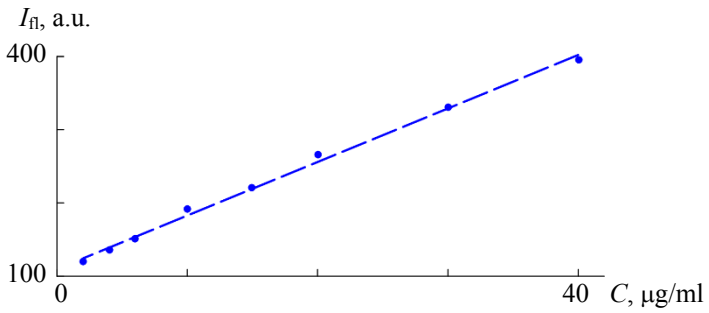

Fig. 2. Calibration plot for azelastine HCl with the proposed method.

Different concentration levels of the drug for the analysis were prepared from independent stock solutions. Assessment of the accuracy of the developed methods was done by spiking the excess drug (50, 100, and 150%) to the pre-analyzed drug solution samples (10 μg/ml). Accuracy was determined as the mean % recovery of the spiked drug concentration (Table 1).

Intra-day precision was investigated by analyzing varying concentrations of azelastine in six independent replicates on the same day (Table 3). Inter-day precision was ascertained from similar determinations carried out on three consecutive days (Table 4). The method was found to be sufficiently precise with %RSD for the intra-day and inter-day precision not exceeding 1.20 and 0.42%, respectively. The calculated intra-day and inter-day data on the precision of the proposed method of analysis are given in Table 1, and no significant variation in the calculated drug concentration was observed on any day. This showed that the method was sufficiently precise for determining the drug concentration.

The LOD and LOQ were calculated using the formulas 3.3 σ/*s* and 10 σ/*s*, respectively, where σ is the standard deviation of the response (calculated from the standard deviation of intercept) and *s* is the slope of the calibration curve. The slopes and intercepts of calibration plots for three sets of fluorescence intensities taken from linearity studies (Table 5) were taken for the calculation of LOD and LOQ values. The LOD and LOQ were found to be 0.1598 and 0.4845 μg/ml, respectively. Further, solutions of the drug having concentrations corresponding to LOD and LOQ values were prepared and analyzed six times ($n = 6$). The %RSD for the results corresponding to both LOD and LOQ were found to be less than 2.8%.

TABLE 3. Fluorescence Intensities in the Intra-day Precision Studies with Pure Drug Azelastine HCl

Concentration, μg/ml	Fluorescence intensity					
	1	2	3	4	5	6
4.0	134.8	134.9	134.7	134.5	134.4	134.6
10.0	189.9	190.1	190.9	190.2	190.5	191.1
30.0	330.9	331.1	330.9	330.5	330.6	330.8

TABLE 4. Fluorescence Intensities in the Inter-day Precision Studies with Pure Drug Azelastine HCl

Day 1			Day 2			Day 3		
4.0 μg/ml	10.0 μg/ml	30.0 μg/ml	4.0 μg/ml	10.0 μg/ml	30.0 μg/ml	4.0 μg/ml	10.0 μg/ml	30.0 μg/ml
132.6	189.6	331.9	134.5	191.6	330.5	134.6	191.5	330.5
134.9	191.6	332.1	133.9	192.9	332.9	133.8	187.3	332.9
133.9	192.1	331.2	134.9	191.6	330.9	133.9	192.6	331.1

TABLE 5. Data for the Calculation of LOD and LOQ

Parameter	Set I	Set II	Set III	Mean	Std. dev.
Slope	7.2974	7.375	7.2268	7.2997**	0.0741
Intercept	109.84	110.32	110.53	110.23	0.3536*

*Standard deviation of the response.
**Mean slope of the calibration plot.

The method was found to be robust, as no significant changes in the fluorescence intensity were observed on carrying out deliberate changes in the method variables, including the excitation wavelength, emission wavelength, and the analyst performing the study. The %RSD in all cases was found to be less than 1.22%. The results from the robustness studies are shown in Table 6. The solutions were found to be stable up to 8 h with %RSD values lower than 2.0%.

The stability indicating potential of the developed methods was evaluated by fortifying a mixture of stress-degraded solutions of azelastine (under conditions of alkaline hydrolysis, alkaline/acidic/neutral photolysis, and oxidative stress) with three known concentrations of the drug to increment the drug concentration by nearly 50, 100, and 150% (final spiked drug concentration 5.0, 10.0, and 20.0 μg/ml, respectively). The original drug concentration in all the stressed solutions was the same (10.0 μg/ml), but due to varying degradation profiles under different conditions, the fluorescence intensities of the solutions were correspondingly reduced. Maximum degradation was noted under alkaline photolytic/hydrolytic conditions (remaining drug concentration 26.4

and 29.5% respectively) followed by oxidative (36.6%), acidic photolytic (49.0%), and neutral hydrolytic conditions (67.9%). Hence, estimation of the spiked drug content in all these solutions was carried out by deducting the fluorescence contribution of the original stressed solution from the observed fluorescence intensity of the spiked solutions. The method afforded excellent recoveries of the added drug in all cases ranging from 95.6 to 99.0% (Table 7), suggesting that the generated degradation products did not significantly interfere with the analytical determination of the drug, and established the stability-indicating nature of the method.

TABLE 6. Robustness of the Proposed Method

Parameter	Change	Fluorescence intensity			Mean	SD	%RSD
Optimized conditions	NA	124.8	125.9	126.9	125.86	1.05	0.83
Excitation λ_{max} (291 nm)	295	110.9	112.6	109.9	111.13	1.36	1.22
Emission λ_{max} (359 nm)	365	115.8	116.8	114.9	115.83	0.95	0.82
Analyst I	Analyst II	123.9	124.6	126.4	124.96	1.28	1.03

TABLE 7. Recovery Studies with the Degraded Samples of Azelastine HCl

Degradation condition	Mean fluor. ^ ±S.D. (%RSD); % Recovery^ ±S.D. (%RSD)	Mean fluorescence intensity^ ±S.D. (%RSD); %Recovery^ ±S.D. (%RSD)		
	Unfortified solution*	Fortified solution**		
		5 µg/ml	10 µg/ml	20 µg/ml
Alkaline hydrolysis	131.5±0.15 (0.11%); 29.5±0.02 (0.70%)	167.3±0.53 (0.32%) 97.9±0.05 (1.13%)	202.5±0.53 (0.26%); 97.1±0.09 (0.95%)	275.9±0.35 (0.13%); 98.8±0.06 (0.32%)
Oxidative	136.7±0.20 (0.14%); 36.6±0.03 (0.74%)	172.2±0.70 (0.41%); 97.3±0.08 (1.71%)	208.4±0.50 (0.24%); 98.2±0.04 (0.42%)	280.5±0.66 (0.24%); 98.7±0.06 (0.34%)
Acidic photolytic	145.8±0.30 (0.21%); 49.0±0.04 (0.85%)	180.9±0.57 (0.32%); 96.2±0.11 (2.37%)	215.7±0.51 (0.23%); 95.7±0.06 (0.65%)	289.9±1.22 (0.42%); 98.6±0.16 (0.81%)
Alkaline photolytic	129.2±0.35 (0.27%); 26.4±0.05 (1.82%)	164.1±0.61 (0.37%); 95.6±0.13 (2.73%)	201.1±0.23 (0.11%); 98.4±0.02 (0.24%)	273.8±0.70 (0.26%); 99.0±0.12 (0.64%)
Neutral photolytic	159.6±0.26 (0.16%); 67.9±0.04 (0.53%)	195.0±0.66 (0.34%); 97.0±0.09 (2.04%)	230.2±0.65 (0.28%); 96.7±0.12 (1.29%)	300.2±0.83 (0.28%); 96.3±0.13 (0.69%)

^Calculated as the mean of the measurements in triplicate (n = 3). The percentage calculated with respect to the original drug.

*The original concentration of each diluted degraded drug solution was 10.0 µg/ml.

**Equal volumes of drug solutions (10, 20, and 40 µg/ml) added to the pre-analyzed diluted degradation drug solution (10 µg/ml).

TABLE 8. Recovery Studies from the Drug Formulation

Conc. taken, µg/ml	Fluorescence intensity	Recovery of the drug
28.0	305.9	26.79
	306.9	26.97
	307.9	27.11
	308.6	27.20
Mean	307.25	27.02
SD	1.30	0.17
%RSD	0.42	0.66

Data from recovery studies of the drug in the marketed nasal spray formulation of azelastine (EZICAS AZ®) are shown in Table 8. The marketed formulation showed recoveries ranging from 95.6 to 97.1% with low % RSD values (0.66%).

Conclusions. A rapid and reliable spectrofluorimetric method has been proposed for the determination of azelastine HCl in its bulk and marketed nasal spray formulation. The method was validated for various

parameters, including sensitivity, reproducibility, precision, accuracy, robustness, and solution stability for ≥8 h. Good recoveries of the drug from bulk samples and the drug formulation suggest that the method is suitable for routine drug analysis without interference from the formulation excipients. The stability indicating nature of the method was suggested by excellent recovery of the spiked drug in various force degraded solutions. Further, studies with various stability samples indicated a high rate of degradation in alkaline, oxidative, and photolytic solutions.

Acknowledgments. We sincerely thank Cipla Ltd., Mumbai (India) for graciously providing pure samples of azelastine HCl.

This work was supported by the University Grants Commission (UGC), New Delhi, India (Grant No. 36/N/296).

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