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Article

Gradient HPLC-DAD Stability Indicating Determination of Miconazole Nitrate and Lidocaine Hydrochloride in their Combined Oral Gel Dosage Form

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The pharmaceutical combination of miconazole nitrate (MZ) and lidocaine hydrochloride (LD) is used in the curative and prophylactic therapy of the oral and gastro-intestinal infections caused by *Candida albicans*. To the best of our knowledge, no attempts have yet been made to assay this combination by any analytical method. A simple and selective high-performance liquid chromatography–diode array detection (HPLC–DAD) stability-indicating method was developed for the simultaneous determination of MZ and LD in their combined formulation. Effective chromatographic separation was achieved using a Zorbax SB-C8 column with gradient elution of the mobile phase composed of 0.05M phosphoric acid and acetonitrile. The gradient elution started with 25% (by volume) acetonitrile, ramped up linearly to 65% in 6 min, then kept constant until the end of the run. The mobile phase was pumped at a flow rate of 1 mL/min. The multiple wavelength detector was set at 215 nm and analytes were quantified by measuring their peak areas. The retention times for LD and MZ were approximately 4.1 and 8.4 min, respectively. The reliability and analytical performance of the proposed HPLC procedure were statistically validated with respect to linearity, ranges, precision, accuracy, selectivity, robustness, detection and quantification limits. Calibration curves were linear in the ranges of 5–100 μg/ml for both drugs with correlation coefficients > 0.999. Both drugs were subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. The proposed method proved to be stability-indicating by the resolution of the two analytes from the related substance and potential impurity (2,6-dimethylaniline) and from the forced-degradation products. The validated HPLC method was applied to the analysis of MZ and LD in the combined oral gel preparation, in which the two analytes were successfully quantified and resolved from the pharmaceutical additives. The proposed method made use of DAD as a tool for peak identity and purity confirmation.

Introduction

Miconazole nitrate (MZ) (Figure 1), chemically known as 1-[(2RS)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl)]-1H-imidazole nitrate (1), is an imidazole antifungal used in the treatment of superficial candidiasis. It may also be given orally as a gel for the treatment of oropharyngeal and intestinal candidiasis (2). Both the British Pharmacopoeia (BP) and the United States Pharmacopeia (USP) describe titrimetric procedures for the assay of the MZ bulk form and high-performance liquid chromatography (HPLC) methods for the assay of various MZ formulations (1, 3). Several methods have been described in the literature for the determination of MZ in its single, multi-component dosage forms or in other complex matrices. These methods include HPLC with ultraviolet (UV) detection (4, 5), HPLC–mass spectrometry (MS) (6), high-performance thin-layer chromatography (HPTLC) (7), capillary electrophoresis (CE) (8), GC with flame-ionization detection (FID) (9, 10), proton nuclear magnetic resonance (11) and several spectrophotometric methods (12–14). Few reports have described the stability-indicating determination of MZ in cream preparations using densitometry (15) and HPLC–diode array detection (DAD) (16).

Lidocaine hydrochloride (LD) (Figure 1), chemically known as 2-(diethyl amino)-*N*-(2,6-dimethylphenyl)acetamide hydrochloride (1), is a local anesthetic with several therapeutic uses including infiltration anesthesia, regional nerve blocks, surface anesthesia, treatment of ventricular arrhythmias and prevention of pain and discomfort from various medical and surgical procedures (2). The BP (1) and the USP (3) suggest several procedures for the assay of LD powder and dosage forms. Most BP procedures depend on titrimetry. HPLC has been described for the assay of the ointment form. On the other hand, HPLC is predominant in the USP monographs of LD and its dosage forms, whereas titrimetric procedures are used for the semisolid topical preparations (ointment and jelly). The quantification of LD in its various drug formulations and/or biological samples has been addressed in numerous reports. Analytical methodology has involved the use of separation techniques such as HPLC-UV (17, 18), HPLC-DAD (19), HPTLC (20), CE with electrochemiluminescence detection (21, 22), CE with mass spectrometric detection (23), GLC with nitrogen phosphorus detector (24) and GC–MS (25). Other analytical techniques have also been adopted, such as derivative spectrophotometry (26, 27), chemometrics-assisted spectrophotometry (28), cyclic and square-wave voltammetry (29) and selective electrode potentiometry (30). Several procedures have been reported describing the stability-indicating analysis of LD, primarily in the presence of its potential impurity 2,6-dimethylaniline. These reports include HPLC (31, 32), HPLC with amperometric detection (33), GC coupled with FID (34), TLC-densitometry (35) and derivative spectrophotometry (35). Forced degradation of LD was attempted by keeping its solution at 70°C for 3 h prior to analysis by HPLC–DAD, in which no degradation peaks were observed for LD (36). Recently, HPLC–DAD procedures have been published describing the stability-indicating assay of some pharmaceutical combinations containing LD (37, 38).

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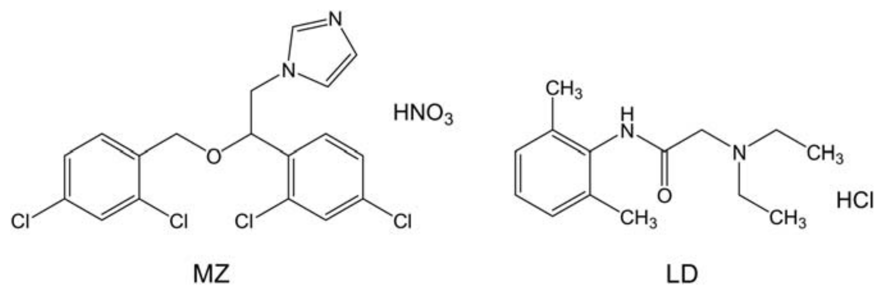

Figure 1. Structures of miconazole nitrate (MZ) and lidocaine hydrochloride (LD).

MZ and LD are co-formulated in an oral gel dosage form used as curative and prophylactic therapy for candidal infection of the oropharyngeal cavity and the gastrointestinal tract. To the best of our knowledge, no reports have been published about the assay of this pharmaceutical mixture. This study describes the development, validation and application of a simple and selective HPLC–DAD procedure for the analysis of this drug combination. Moreover, the proposed procedure was tested for its stability-indicating properties by the resolution of LD from its potential impurity (2,6-dimethylaniline), and the separation of both drugs from their forced degradation products.

Experimental

Instrumentation

The HPLC–DAD system consisted of Agilent 1200 series (Agilent Technologies, Santa Clara, CA) (quaternary pump, vacuum degasser and diode array and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation software. A Rheodyne manual injector with a 20-mL loop was used. The column used was Zorbax SB-C8 (4.6 × 250 mm, 5 μm particle size) (Agilent).

Materials

LD was supplied by Alexandria Company for Pharmaceuticals (Alexandria, Egypt). MZ was donated by Amriya Pharmaceutical Industries (Alexandria, Egypt). 2,6-Dimethylaniline (Sigma-Aldrich, St. Louis, MO), HPLC-grade acetonitrile (Scharlau Chemie S.A., Sentmenat, Spain), HPLC-grade methanol (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), analytical grade ortho-phosphoric acid, hydrochloric acid, sodium hydroxide, 30% hydrogen peroxide and high purity distilled water were used. The pharmaceutical preparation assayed in the study is Micoban oral gel (Amriya Pharmaceutical Industries, Alexandria, Egypt) labeled to contain 25 mg MZ and 6.6 mg LD per gram of gel, in addition to the inactive ingredients: methyl 4-hydroxybenzoate (methyl paraben) and saccharin sodium.

General procedure

A gradient mobile phase system consisting of (A) 0.05 M phosphoric acid and (B) acetonitrile was used. The separation was achieved with a linear gradient program as follows: 25% v/v B at zero time; from 0 to 6 min, ramp up to 65% v/v B; from 6 to 10 min, holding 65% v/v B. After 10 min, the gradient program was returned to the initial conditions and the analytical column was reconditioned for 3 min. The flow rate was 1.0 mL/min. The injection volume was 20 μL. The eluent was monitored by the DAD from 190 to 400 nm, and chromatograms were extracted at 215 nm. All determinations were performed at 25°C.

MZ stock solution (500 μg/ml) and LD stock solution (500 μg/ml) were prepared in HPLC-grade methanol. The working solutions were prepared by dilution of the stock solutions with distilled water + methanol (1:1) to reach the concentration range 5–100 μg/ml for both MZ and LD. Triplicate injections were made for each concentration and chromatographed under the previously described LC conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration graphs.

Assay of the pharmaceutical dosage form

A portion of the oral gel preparation (1.0 gm equivalent to 25 mg MZ and 6.6 mg LD) was weighed and extracted into HPLC-grade methanol with the aid of sonication for 10 min, then filtered into a 100-mL volumetric flask. The filtrate was completed to volume with HPLC-grade methanol. Aliquots of the dosage form methanolic extract were diluted with distilled water + methanol (1:1) to obtain final concentrations within the specified ranges, then treated as described previously. Recovered concentrations were calculated from the corresponding calibration graphs. For standard addition assay, sample solutions were spiked with aliquots of standard solutions of both compounds to obtain total concentrations within the previously specified ranges, then treated as described previously. Recovered concentrations were calculated by comparing the analyte response with the increment response attained after addition of the standard.

Forced degradation and stability-indicating study

A stock solution of 2,6-dimethylaniline (1000 μg/ml) was prepared in HPLC-grade methanol. An aliquot of this stock solution was added to the two drugs under analysis and the solution was diluted to volume with distilled water + methanol (1:1). This mixture was chromatographed under the previously described LC conditions. Forced degradation studies were carried out on MZ and LD standards and their commercial oral gel extract according to the conditions described in the following.

(a) Acidic and basic conditions: MZ and LD solutions were treated with 1 mL of 1M HCl or 1M NaOH. A set of solutions was kept at room temperature for 24 h, and another set was placed in a water bath at 100°C for 1 h under reflux. After the specified time, all solutions were neutralized by adjusting the pH to 7.0 and then diluted with distilled water + methanol (1:1) to reach a final concentration of 50 μg/ml of each compound. The same procedure was applied to aliquots of the dosage form methanolic extract.

(b) Oxidation with H_2O_2: MZ and LD solutions were treated with 0.5 mL of hydrogen peroxide 5%. A set of solutions was kept at room temperature for 24 h and another was placed in a water bath at 80°C for 1 h. After the specified time intervals, the solutions were diluted with distilled water + methanol (1:1) to reach a final concentration of 50 μg/ml of each compound. Aliquots of the dosage form methanolic extract were similarly treated.

(c) UV degradation: MZ and LD solutions (50 μg/ml) were subjected to UV irradiation at 254 nm for 6 h. Solutions prepared by dilution of aliquots of the dosage form methanolic extract were similarly treated.

(d) Dry heat degradation: An amount of each drug powder (100 mg) was kept in an oven at 90°C for 7 h. After the specified time, each powder was dissolved in methanol, and aliquots of these methanolic stocks were diluted with distilled water + methanol (1:1) to reach a final concentration of 50 μg/ml of each compound.

After the previous treatments, solutions were filtered with a 0.45 μm filtration disk before injection to the column.

Results and Discussion

Optimization of chromatographic conditions

The simultaneous determination of MZ and LD using conventional spectrophotometry is not feasible. This can be attributed to the strong overlap between their absorption spectra. Another complicating factor is the presence of several UV-absorbing inactive ingredients in MZ-LD combined dosage form. In such mixtures, only a separation technique can be successful to achieve the goal. A gradient LC method coupled with diode array detector was developed to provide a suitable procedure for the rapid and reliable quality control analysis of MZ and LD in their combined pharmaceutical preparation. The most important aspect in LC method development is the achievement of sufficient resolution with acceptable peak symmetry in a reasonable analysis time. For optimization of the stationary phase, several reversed phase columns [Zorbax SB-C8 (4.6 × 250 mm), Zorbax SB-C18 (4.6 × 250 mm), Zorbax Eclipse XDB-C18 (4.6 × 150 mm) and Waters Symmetry C18 (3.9 × 150 mm)] were tested. All columns gave satisfactory resolution of the two analytes; however, less sharp and more retained MZ peaks were observed with C18 columns, probably due to its dibenzenoid structure, which shows strong affinity to the C18 surface. The Zorbax SB-C8 column was found to be optimum; hence, it became the column of choice for this study. Several mobile phases were evaluated using various proportions of different aqueous phases and organic modifiers. The best mobile phase combination was 0.05M phosphoric acid solution and acetonitrile. Methanol was tried as an organic modifier and phosphoric acid solution was substituted by other aqueous phases such as water, acetic acid solution or sodium monohydrogenphosphate solution. In these trials, MZ suffered from increased retention times and some chromatograms showed broader peaks; additionally, poor separation was observed between the analytes' peaks and those of the additives of the pharmaceutical formulation. Isocratic elution of different proportions of 0.05M phosphoric acid and acetonitrile did not provide satisfactory separation, primarily due to the inadequate resolution between the parent drugs (MZ and LD) and the related substance (2,6-dimethylaniline), in addition to the forced-degradation products, especially those resulting from oxidation with H_2O_2; therefore, gradient elution was applied. Several gradient programs were tried and the best compromise between adequate resolution and reasonable retention times was achieved by using a gradient system starting with 25% (by volume) acetonitrile ramped up linearly to 65% in 6 min, then kept at this percentage afterwards. Flow rate was kept constant at 1.0 mL/min throughout the run.

The multiple wavelength detector offers the advantage of measuring each analyte at its maximum wavelength, thus improving sensitivity. In addition, DAD enhances the power of HPLC and is an elegant option for assessing method specificity and peak purity by comparison of recorded spectra during peak elution. Quantification was achieved using DAD based on peak area measurement. Both MZ and LD are considered weak UV-absorbing compounds. They exhibit considerable absorbance only in the short UV region (below 230 nm); consequently, 215 nm was found suitable to record all chromatograms in this study and to quantify both MZ and LD.

The previously described chromatographic conditions showed good separation between MZ and LD within acceptable run time. Figure 2 shows a typical chromatogram for the separation of the two analytes, LD and MZ, eluted at retention times 4.11 ± 0.023 and 8.40 ± 0.061 min, respectively. Column performance (apparent efficiency) can be expressed by the number of theoretical plates (N), which equals approximately 10700 and 27200 for LD and MZ, respectively.

Analytical performance of the proposed method

Linearity and concentration ranges

The linearity of the proposed HPLC procedure was evaluated by analyzing a series of different concentrations for each

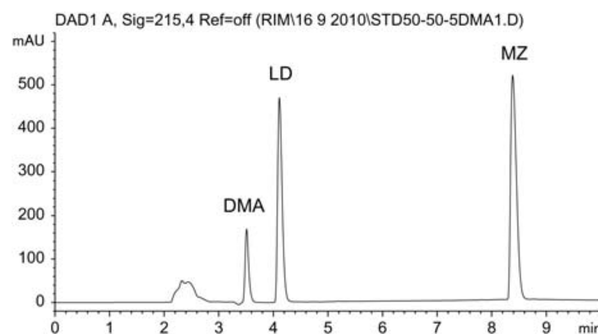

Figure 2. HPLC chromatogram of 20 μL injection of a mixture containing 50 μg/mL MZ, 50 μg/mL LD and 5 μg/mL 2,6-dimethylaniline (DMA).

Table I
Analytical parameters for the determination of MZ – LD mixture using the proposed HPLC-DAD method

Parameter	MZ (215 nm)	LD (215 nm)
Concentration range (μg/mL)	5 – 100	5 – 100
Intercept (a)	-26.83	23.75
S_a^a	48.82	27.01
Slope (b)	78.59	51.65
S_b^b	0.83	0.46
RSD% of the slope	1.06	0.89
Correlation coefficient (r)	0.99978	0.99984
$S_{y/x}^c$	63.92	35.36
LOD[d] (μg/mL)	0.38	0.47
LOQ[e] (μg/mL)	1.26	1.55

[a] Standard deviation of the intercept.
[b] Standard deviation of the slope.
[c] Standard deviation of residuals.
[d] Limit of detection.
[e] Limit of quantification.

compound. The linear regression equations were generated by least squares treatment of the calibration data. Under the optimized conditions described previously, the measured peak areas were found to be proportional to concentrations of the analytes. Table I presents the performance data and statistical parameters, including linear regression equations, concentration ranges, correlation coefficients, standard deviations of the intercept (S_a), slope (S_b) and standard deviations of residuals ($S_{y/x}$). Regression analysis shows good linearity, as indicated from the correlation coefficient values (>0.9997). In addition, deviation around the slope can be further evaluated by calculation of the percentage relative standard deviation (RSD%) of the slope (S_b%), which were found to be less than 1.1 %.

Detection and quantification limits

According to the pharmacopeial recommendations (1, 3), the limit of detection (LOD) is defined as the concentration that has a signal-to-noise ratio of 3:1, while for limit of quantification (LOQ), the ratio considered is 10:1. The LOD and LOQ values for the studied analytes were calculated and presented in Table I.

Precision and accuracy

The within-day (intra-day) precision and accuracy for the proposed method were studied at three concentration levels for each compound using three replicate determinations for each concentration within one day. Similarly, the between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations for each compound using three replicate determinations repeated on three days. Recoveries were calculated using the corresponding regression equations, which were satisfactory. The RSD% and percentage relative error (E_r%) did not exceed 1.5%, proving the high repeatability and accuracy of the developed method for the estimation of the analytes in their bulk form (Table II).

Selectivity and specificity

Method selectivity was examined by preparing several laboratory-prepared mixtures of the two compounds at various concentrations within the linearity ranges mentioned in Table I. The laboratory-prepared mixtures were analyzed according to the previously described procedure. The analysis results, including RSD% and E_r% values shown in Table III were satisfactory, thus validating the selectivity, precision and accuracy of the developed method and demonstrating its capability to resolve and quantify the analytes in different ratios. Specificity is defined as the ability to unequivocally access the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components (3), and this will be shown in the following sections of this study.

Table II
Precision and accuracy for the determination of MZ and LD in bulk form using the proposed HPLC-DAD method

Analyte	Nominal value (μg/mL)	Within-day			Between-day		
		Found ± SD[a] (μg/mL)	RSD(%)[b]	E_r(%)[c]	Found ± SD[a] (μg/mL)	RSD(%)[b]	E_r(%)[c]
MZ	20	20.12 ± 0.13	0.65	0.60	20.20 ± 0.19	0.94	1.00
	50	49.68 ± 0.24	0.48	-0.64	49.43 ± 0.43	0.87	-1.14
	80	80.92 ± 0.43	0.53	1.15	80.47 ± 0.73	0.91	0.59
LD	20	19.79 ± 0.11	0.56	-1.05	19.87 ± 0.16	0.81	-0.65
	50	50.34 ± 0.39	0.78	0.68	50.47 ± 0.43	0.85	0.94
	80	80.70 ± 0.49	0.61	0.88	80.86 ± 0.82	1.01	1.08

[a] Mean ± standard deviation for three determinations.
[b] % Relative standard deviation.
[c] % Relative error.

Table III
Determination of MZ – LD laboratory-prepared mixtures using the proposed HPLC-DAD method

Nominal value (μg/mL)		Found ± SD[a] (μg/mL)		RSD(%)[b]		E_r(%)[c]	
MZ	LzD	MZ	LD	MZ	LD	MZ	LD
100	10	99.21 ± 1.05	9.88 ± 0.09	1.06	0.91	-0.79	-1.20
100	20	99.06 ± 0.90	19.86 ± 0.19	0.91	0.96	-0.94	-0.70
80	20	79.63 ± 0.62	19.84 ± 0.22	0.78	1.11	-0.46	-0.80
40	20	40.31 ± 0.38	19.67 ± 0.16	0.94	0.81	0.78	-1.65
40	40	39.52 ± 0.33	40.36 ± 0.31	0.84	0.77	-1.20	0.90
20	40	20.17 ± 0.23	39.86 ± 0.44	1.14	1.13	0.85	-0.35
20	80	20.21 ± 0.18	79.47 ± 0.60	0.89	0.76	1.05	-0.66

[a] Mean ± standard deviation for five determinations.
[b] % Relative standard deviation.
[c] % Relative error.

Robustness

Robustness was examined by evaluating the influence of small variations in different conditions, such as concentration of phosphoric acid solution (± 0.005M), ratio of acetonitrile in the gradient program (± 2%, *v*/*v*), source of acetonitrile (Scharlau Chemie S.A., Spain or SDS, France), working wavelengths (± 1 nm) and flow rate (± 0.1 mL/min). These variations did not have any significant effect on the measured responses or the chromatographic resolution. RSD% for the measured peak areas using these variations did not exceed 3%.

Stability of solutions

The stability of standard working solutions in addition to sample solutions in the diluting solvent [water + methanol (1:1)] was examined and no chromatographic changes were

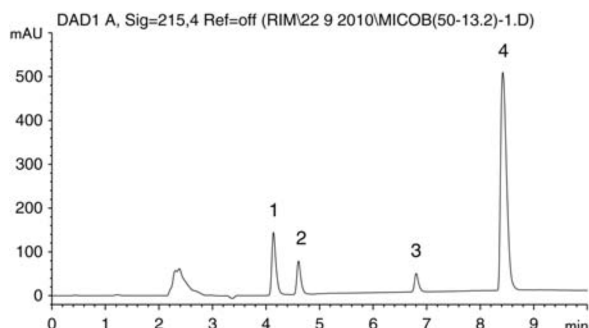

Figure 3. HPLC chromatogram of 20 μL injection of a mixture containing 50 μg/mL MZ and 13.2 μg/mL LD obtained from Micoban® oral gel (1 = LD, 2 = saccharin sodium, 3 = methyl paraben and 4 = MZ).

Table IV
Application of the proposed HPLC-DAD method to the analysis of MZ – LD mixture in its pharmaceutical preparation

	External Standard		Standard Addition	
	MZ	LD	MZ	LD
%Recovery ± SD[a]	98.74 ± 0.83	99.03 ± 0.91	99.25 ± 0.89	99.35 ± 0.96
RSD%[b]	0.84	0.92	0.90	0.97

[a] Mean ± standard deviation for five determinations.
[b] % Relative standard deviation.

observed within 24 h at room temperature. Also, the stock solutions were stable for at least two weeks when stored in refrigeration at 4°C. Retention times and peak areas of the drugs remained unchanged and no significant degradation was observed during these periods.

Analysis of pharmaceutical dosage form

The developed HPLC–DAD procedure was applied for the assay of this drug combination in its pharmaceutical formulation available in the local market (Micoban oral gel). Sample preparation included only a simple extraction procedure using HPLC-grade methanol as solvent without prior separation of the active pharmaceutical ingredients from the inactive additives. Aliquots of the methanolic extract were diluted with distilled water + methanol (1:1) to reach the specified concentration ranges, then injected to the column. A representative chromatogram obtained from the dosage form solutions is shown in Figure 3. The two active ingredients eluted at their specific retention times (4.1 and 8.4 min for LD and MZ, respectively). The inactive ingredients of the formulation appeared as well-resolved peaks at retention times 4.61 and 6.80 min. The peak eluting at 4.61 min shows an absorption spectrum matching the reported spectrum of saccharin sodium, whereas the UV spectrum for the peak at 6.80 min is identical to that reported for methyl paraben (39). The DAD enables peak purity verification of the two analytes in which no signs of co-elution from any of the inactive components were detected. Recoveries were calculated using both external standard (calibration curves) and standard addition methods. The assay results revealed satisfactory accuracy and precision as indicated from percent recovery, SD and RSD% values (Table IV). It is evident from these results that the proposed method is applicable to the analysis of MZ and LD in their combined commercial formulation with minimum sample preparation and satisfactory level of selectivity, accuracy and precision.

Stability-indicating aspects

The optimized HPLC method was applied to test the chromatographic behavior of LD-related substance 2,6-dimethylaniline (DMA) and the products of forced degradation of both analytes. DMA eluted at retention time 3.51 min, as shown in Figure 2. Resolution (R_s) is a measure of the degree of separation between adjacent peaks. A value of 1.5 for resolution implies a complete separation of any two consecutive peaks (1). Resolution was calculated between the two adjacent peaks of DMA and LD and it was found to be 4.41, which revealed an excellent baseline separation between the parent drug and its potential impurity.

Forced degradation studies can help identify the likely degradation products of a drug substance, which can in turn help to establish the degradation pathways and intrinsic stability of the molecule and validate the stability-indicating power of the proposed analytical procedure (40). Forced degradation experiments were carried out on MZ and LD mixtures to produce the possible relevant degradants and test their chromatographic behavior using the developed method. Hydrolytic (using either 1M HCl or 1M NaOH solutions) and oxidative degradation (using 5% H_2O_2) were conducted at both ambient temperature and with the aid of heating. No signs of degradation of either MZ or LD were observed in acidic or basic conditions. The peaks of both analytes appeared at their specific retention times with areas identical to those of standards of the same concentrations; additionally, the MZ-LD mixture chromatograms after exposure to forced acidic and basic conditions did not show any extra peaks (Figures 4A and 4B). Both drugs were found to be stable to oxidative degradation at room temperature after 24 h; however, a decrease in peak areas of both analytes and appearance of new peaks for degradation products were clearly noticed after heating with H_2O_2 at 80°C for 1 h (Figure 4C). For MZ, approximately 18% degradation was observed, and two minor degradation products peaks eluted before the parent drug peak at retention times 7.88 and 8.26 min. On the other hand, LD showed only approximately 6% degradation and a well-resolved extra peak eluted after the parent drug at retention time 4.73 min (Figure 4C). Hydrogen peroxide reacts with tertiary amines to form tertiary amine oxides (41). In the case of MZ, two products can be formed because it contains two tertiary amine groups susceptible to oxidation, whereas LD has only one tertiary nitrogen. A proposed pathway for the oxidation of both analytes by H_2O_2 is illustrated in Figure 5. No degradation was observed after exposure of a mixture solution to UV light for 6 h; additionally, the mixture chromatogram after stress photolytic exposure did not show any extra peaks (Figure 4D). The stability of MZ and LD to stress photolytic conditions can be explained by the fact that both drugs are weak UV absorbers.

Similar forced degradation experiments were carried out on aliquots of the combined dosage form extract. As mentioned earlier, no signs of degradation of the active drugs were noticed in acidic or basic conditions. This was confirmed by the peak areas, which were identical to those of undegraded

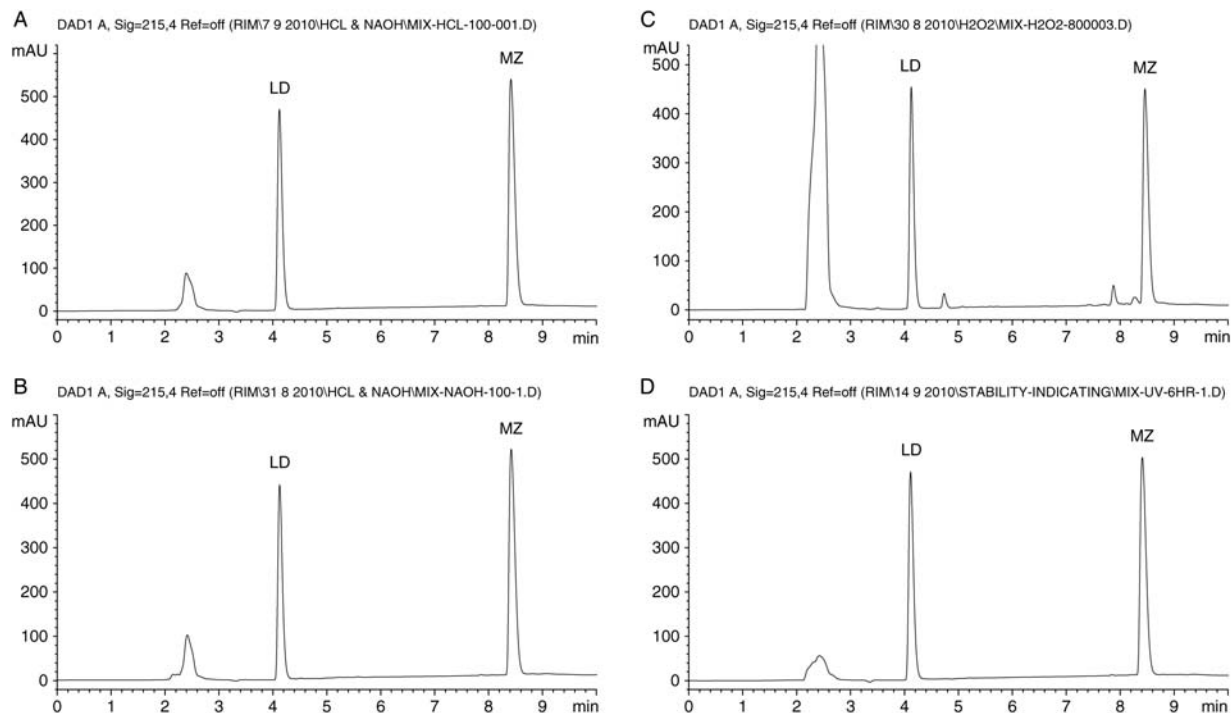

Figure 4. HPLC chromatograms of mixtures of 50 μg/mL MZ and 50 μg/mL LD after exposure to acid degradation (1 M HCl/100°C for 1 hr) (A), base degradation (1 M NaOH/100°C for 1 h) (B), oxidative degradation (5% H_2O_2/80°C for 1 h) (C) and UV at 254 nm for 6 h (D).

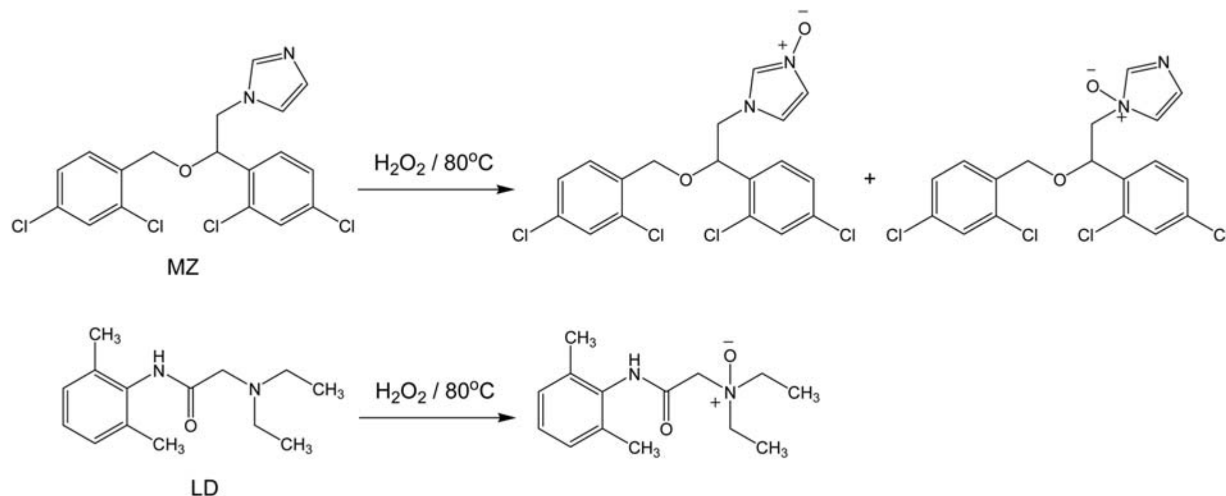

Figure 5. Proposed reaction pathway for the oxidation of MZ and LD with hydrogen peroxide at 80°C.

solutions of the same concentrations. However, an obvious change was noticed for the inactive ingredients' peaks. Acidic degradation resulted in decreased areas for these peaks at 4.6 and 6.8 min; additionally, new minor peaks appeared at retention times 3.35, 3.56, 6.25 and 7.88 min (Figure 6A). Basic degradation resulted in complete disappearance of the inactive ingredient peak at 6.8 min, while that at 4.6 min remained without obvious change. No extra peaks were observed in the obtained chromatogram (Figure 6B). Oxidative degradation of the pharmaceutical dosage form produced similar observations to those obtained with the standard mixture (lower peak area values for MZ and LD, and the evolution of three resolved degradation product peaks at retention times 4.74, 7.84 and 8.24 min); additionally, no signs of change were observed for the inactive ingredient peaks at 4.6 and 6.8 min. Figure 6C shows the chromatogram of the dosage form solution after treatment with 5% H_2O_2 / 80°C for 1 h. Photolytic degradation of the dosage form solution revealed intact active and inactive ingredients without any apparent changes in peak areas or extra peaks (Figure 6D).

Dry heat forced degradation of both analytes revealed stability of the drugs after heating at 90°C for 7 h. After the specified

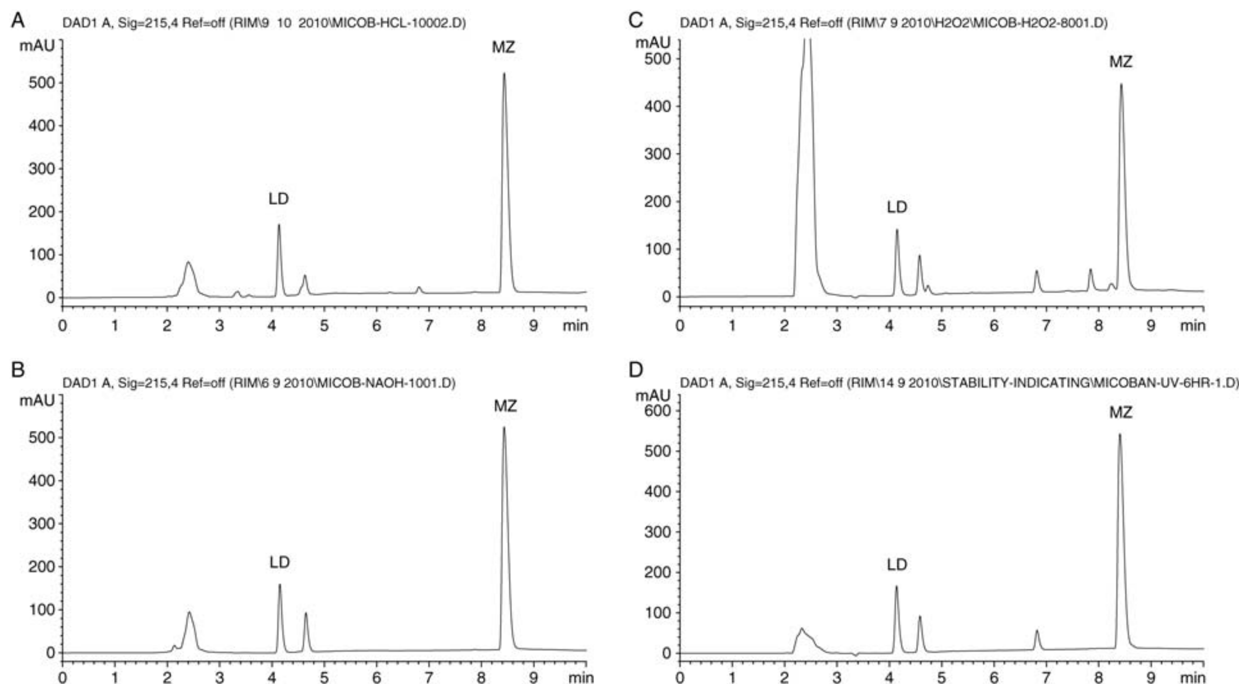

Figure 6. HPLC chromatograms of mixtures of 50 μg/mL MZ and 13.2 μg/mL LD obtained from Micoban® oral gel after exposure to acid degradation (1 M HCl/100°C for 1 h) (A), base degradation (1 M NaOH/100°C for 1 h) (B), oxidative degradation (5% H_2O_2/80°C for 1 h) (C) and UV at 254 nm for 6 h (D).

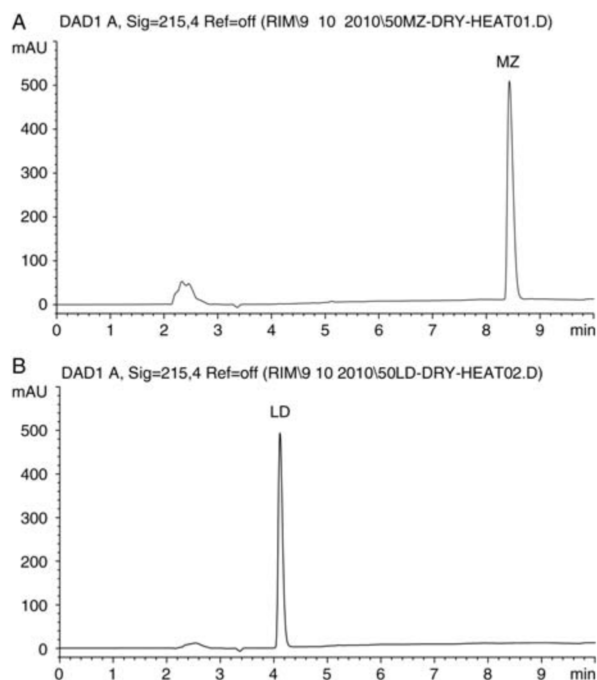

Figure 7. HPLC chromatograms of 50 μg/mL MZ (A) and 50 μg/mL LD (B) after exposure to dry heat degradation by heating at 90°C for 7 h.

time, solutions were prepared from the powders, and peaks of both MZ and LD appeared at their specific retention times with areas identical to those of standards of the same concentrations; additionally, no extra peaks were observed in the chromatograms (Figures 7A and 7B).

Peak purity test results obtained from DAD confirm that MZ and LD peaks are homogenous and pure in all the analyzed samples subjected to forced degradation conditions.

Conclusion

In this study, a simple, selective and reliable gradient elution HPLC–DAD procedure was developed for the assay of MZ and LD in their pharmaceutical combination. To our present knowledge, no attempts have yet been made to assay this mixture in pharmaceutical formulations by any analytical methodology. In the study, both analytes were subjected to forced degradation using several stress conditions. The analytes were found stable to hydrolysis, dry heat and UV conditions, although slight degradation resulted upon applying oxidative conditions. The most important features in the proposed method are its specificity and stability-indicating merits. Both drugs were analyzed in the presence of the potential impurity (2,6-dimethylaniline), forced degradation products and the dosage form excipients. Reliability was guaranteed by testing various validation parameters, which showed good performance of the developed procedure. The developed method made use of DAD as a tool for peak identity and purity confirmation; however, it can be adapted to conventional HPLC with UV detection, which is the most popular in quality control laboratories. Therefore, the proposed method can be recommended for routine analysis and checking quality during stability studies of the two drugs.

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