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Development and validation of a simple stability-indicating high performance liquid chromatographic method for the determination of miconazole nitrate in bulk and cream formulations

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ABSTRACT

A simple and stability-indicating high performance liquid chromatographic method was developed and validated for the determination of miconazole nitrate in bulk and cream preparations. The extraction step for cream samples consisted in a warming, cooling and centrifugation procedure that assures the elimination of the lipophilic matrix component, in order to avoid further precipitation in the chromatographic system. Separation was achieved on a ZORBAX Eclipse XDB – C18 (4.6 mm × 150 mm, 5 μm particle size) column, using a mobile phase consisting of water, methanol and acetonitrile, in a flow and solvent gradient elution for 15 min. The column was maintained at 25 °C and 10 μL of solutions were injected. UV detection was performed at 232 nm, although employment of a diode array detector allowed selectivity confirmation by peak purity evaluation. The method was validated reaching satisfactory results for selectivity, precision and accuracy. Degradation products in naturally aged samples could be simultaneously evaluated, without interferences in the quantitative analysis.

1. Introduction

Miconazole, 1H-imidazole,1-[2-(2,4-dichlorophenyl)-2-[(2,4-dicholophenyl)methoxy]ethyl]-(±) (Fig. 1) is an antimycotic-imidazole derivative [1]. With a wide activity spectrum, it is endowed with a powerful activity against dermatophytes and *Candida albicans*, as well as against several Gram-positive germens [2].

The generally accepted mode of action of azole antifungals is the inhibition of 14-alpha-lanosterol demethylase, a key enzyme in ergosterol biosynthesis, resulting in depletion of ergosterol and accumulation of toxic 14-alpha-methylated sterols in the membrane of susceptible yeast species [3].

Miconazole has been extensively applied in the management of dermal, oral, and vaginal mycosis. Currently, it is used in a variety of pharmaceutical formulations such as injections, tablets, oral gels, creams, ointments, topical powders and vaginal suppositories. The most usual application forms include creams, ointments or gels at 2.0% concentration level, alone or associated with anti-inflammatory steroids, or other antimicrobials such as gentamicine for the treatment of dermatitis. Diaper dermatitis, a common dermatologic disorder of infancy, frequently associated with *C. albicans* infections, is currently treated with ointments containing 0.25% miconazole nitrate [3]. Moreover, mucoadhesive buccal patches containing miconazole nitrate have been developed, and very recently an extended release miconazole bioadhesive buccal tablet was reported to be effective in the treatment of oropharyngeal candidiasis [4]. Pessaries containing a combination of metronidazol and miconazol nitrate are used in treatment of vulvovaginal infections [4].

USP [1] (United States Pharmacopoeia) and British [5] Pharmacopoeia methods for the assay of miconazole nitrate and related substances or degradation products in bulk and pharmaceutical dosage forms are diverse. The bulk material assay is performed by a potentiometrical titrimetry in both pharmacopoeias, whereas related compounds are evaluated through an isocratic high performance liquid chromatography (HPLC) method. This method employs 0.2 mol L^{-1} ammonium acetate, methanol, and acetonitrile (38:32:30) as the mobile phase at a flow rate of 2 mL min^{-1} and a 4.6 mm × 100 mm column containing 3 μm C18 packing. Under these conditions, the chromatographic analysis requires

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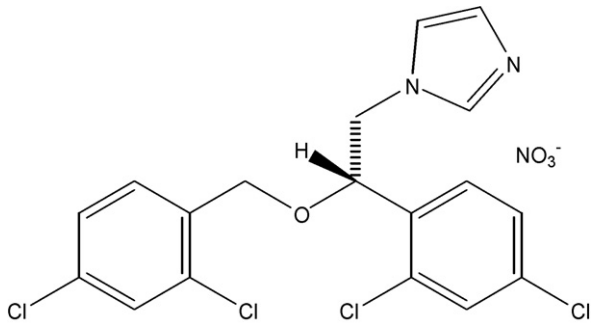

Fig. 1. Structural formulae for miconazole nitrate (MW = 479.1).

a considerable time for equilibration and for obtaining results (ca. 30 min). On the other hand, the same method is described in British Pharmacopoeia for the simultaneous determination of related substances, such as for the assay of miconazole in cream preparations. However, a gradient of 0.01 mol L^{-1} ammonium dihydrogen orthophosphate pH 6.0, tetrahydrofuran and acetronitrile is employed in the latter method for assaying miconazole and hydrocortisone in creams. The 30th edition of the USP suggests an HPLC based method to analyze cream and vaginal ovule formulations containing miconazole nitrate, which employs a mobile phase consisting of triethylamine and phosphoric acid in a mixture of acetonitrile, tetrahydofuran and methanol. When analysing injections, the mobile phase in the USP method is an ammonium acetate buffer in acetonitrile and methanol, whereas a GC method is suggested for powders. To the best of our knowledge, no analytical chromatographic methods were reported in the literature for the routine quantitative assay of miconazole nitrate in bulk form, and only a limited number of methods for its assay in pharmaceutical dosage forms have been presented. Colorimetry [6], derivative spectrophotometry [7,8], HPLC [9] and magnetic resonance spectroscopy [10] were applied in tablets, powders and ovules containing miconazole nitrate. On the other hand, solid phase extraction (SPE) prior HPLC with post-column photochemical reaction [11] and supercritical fluid extraction followed by derivative spectroscopy [12] were described for cream preparation analysis.

Cream analysis generally represents a difficult task for analysts, essentially due to the complexity of the base cream or matrix in which the analyte of interest is immersed. It usually contains vaseline, paraffine, cetoestearilic alcohol or other lipophilic components which are poorly soluble in the solvents commonly used in HPLC. For this reason, the assay method requires an extractive procedure prior to the chromatographic step in order to ensure the elimination of interfering components, avoiding the precipitation in the chromatographic system.

In the present report, we present a strategy which allows for the simultaneous quantification and stability evaluation of miconazole nitrate in bulk and cream formulations through a simple, rapid, precise, accurate and specific HPLC-UV method with an efficient and extremely easy extractive procedure. It is interesting to note that a reversed-phase HPLC method for determination of econazole nitrate in cream formulations has been recently developed using miconazole nitrate as an internal standard [13]. However, in the presently proposed method, the separation was achieved in a shorter retention time and consuming a lower amount of solvent. On the other hand, the results obtained in the validation step suggest that it is not necessary to employ an internal standard, simplifying the work. Moreover, in comparison with the methods described in the literature, our chromatographic system needs a simpler mobile phase composition and no buffer, reaching excellent resolution and peak symmetry.

It is internationally admitted that analytical measurements should be made using methods which have been tested to ensure they are fit for the purpose. In this regard, method validation is an important requirement in the practice of chemical analysis, and it should be an integral part of any good analytical practice. Recommendations provided for scientific organizations such as EURACHEM (European Analytical Chemistry) [14,15] and IUPAC (International Union Of Pure and Applied Chemistry) [16,17] can be considered in this way. Moreover, for pharmaceutical analysis, guidelines from the USP [1], ICH [18] (International Conference on Harmonisation), and the FDA [19,20] (Food and Drug Administration) provide a framework for performing such validations. Taking into account these considerations, the proposed methodology has been extensively validated.

2. Experimental

2.1. Chemicals and reagents

A miconazole nitrate working standard was prepared in our laboratory from a raw material, which was characterized and assayed according to specifications of the 30th edition of the USP. Miconazole nitrate bulk material, creams containing (i) 2.0 g% p/p of miconazole (nitrate) or (ii) 2.0 g% of miconazole (nitrate), 0.1% of betamethasone (17-valerate) and 0.1% gentamicine (sulphate), and the base cream utilized in the formulation, were all obtained from LAFORMED SA, Formosa, Argentina. The base cream formulation includes liquid and solid paraffin, cetostearyl alcohol, benzyl alcohol, cetomacrogol 1000, monobasic sodium phosphate, phosphoric acid, propylene glycol, methylparaben, propylparaben and triethanolamine. Raw material of all this compounds was provided by LAFORMED SA (Formosa, Argentina). Methanol and acetonitrile (HPLC grade) were purchased from Aberkon Quimica, Argentina. Glacial acetic acid (analytical grade) was purchased from Laboratorios Cicarelli, Argentina, while purified HPLC grade water was obtained from a Milli-Q® system (Millipore, Milford, MA, USA). Acidic methanol was prepared by diluting glacial acetic acid in methanol to obtain a final concentration of 0.1% (v/v).

2.2. HPLC instrumentation and conditions

The HPLC system was an Agilent 1100 Series equipped with a quaternary pump, membrane degasser, thermostated column compartment, autosampler and diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). For data acquisition and processing, the Chemstation version B 0103 was used. The HPLC column was a ZORBAX Eclipse XDB – C18 (4.6 mm × 150 mm, 5 μm particle size), Agilent Technologies. The chromatographic separation was performed using a mobile phase consisting of water, methanol and acetonitrile, in a flow and solvent gradient elution of 15 min (see Table 1). Solvents were filtered through 0.45 μm pore Nylon filter before use. The column was maintained at 25 °C and an injection volume of 10 μL was used. The photodiode array detector was set at 232 (4) nm with reference in 360 (100) nm and used in scan mode in the range of 200–500 nm for selectivity and stability studies. Peak purity was evaluated from spectral analysis performed with the instrument software.

Table 1
Gradient program used for the separation of miconazole.

Time (min)	Water (%)	Methanol (%)	Acetonitrile (%)	Flow rate (mL min^{-1})
0.0	30	30	40	1.0
2.0	20	35	45	1.0
4.5	20	35	45	1.0
7.0	10	40	50	1.5
10.0	30	30	40	1.0

2.3. Stock and standard solutions

A stock standard solution of miconazole nitrate 11.0 mg mL^{-1} was prepared by exactly weighing and dissolving a portion of the working standard in HPLC grade methanol. This solution, which proved to be stable for a period of 2 month, was stored at 4 °C and protected against light in a refrigerator. The latter solution was left to attain room temperature before use. Working standard solution was prepared daily by dilution of appropriate amount of the stock standard solution in water:methanol:acetonitrile (30:30:40) reaching a final concentration of 0.53 mg mL^{-1}.

2.4. Cream pre-treatment

Portions of ca. 2.2 g of previously well-homogenised cream samples were accurately weighed in 250 mL vessels and 15 mL of acidic methanol were added. Vessels were covered by a watch glass and placed in a water bath at 90 °C with agitation and kept there until the cream components were melted. Afterwards, they were removed from the bath, vigorously shaken for 5 min in a magnetic stirrer plate and kept at room temperature until the specimen resolidified. The heating–shaking–cooling procedure was repeated twice to guarantee the complete solubilization and extraction of miconazole nitrate from the matrix components. The obtained suspensions were then transferred to 25.0 mL volumetric flask, washing the vessels with acidic methanol aliquots that were collected in the same flasks. Suspensions were then made up to volume with the same solvent and 10.0 mL of them were placed in centrifuge tubes with caps. The tubes were kept at −20 °C in freezer during 20 min and then centrifugated at 2000 × *g* for 15 min. This procedure assures the complete precipitation of the lipophilic components of the base cream, whereas miconazole nitrate remains soluble in acidic methanol. After that, 3.0 mL of the supernatants (at room temperature) were transferred to a 10.0 mL flask and made up to volume with a mix of water:acetonitrile (30:40) to achieve a final composition of solvents equal to the initial composition of the mobile phase. The solutions were poured again in new caped centrifuge tubes and kept at −20 °C in freezer for 20 min. Remaining lipophilic components, completely precipitated in this step, were separated by centrifugation at 2000 × *g* for 15 min. Finally, suitable amounts of the solutions, containing about 0.53 mg mL^{-1} of miconazole nitrate, were filtered through a 0.45 μm nylon filter into an injector vial.

2.5. Validation samples

2.5.1. Matrix and excipient solutions

A portion of base cream (blank) of ca. 2.2 g, containing neither miconazole nitrate nor other active ingredients, was processed in the same manner as indicated above for cream pre-treatment.

Cetostearyl alcohol, benzyl alcohol, propylene glycol, methylparaben, propylparaben and triethanolamine solutions 0.5 mg mL^{-1} were prepared in methanol.

2.5.2. Linearity standard solutions

Adequate amounts of the stock standard solution of miconazole nitrate were diluted to obtain solutions with the following concentrations: 0.26, 0.40, 0.53, 0.66 and 0.79 mg mL^{-1}, all of them covering the range from 50.0 to 150.0% of the expected concentration of the analyte in the assay solution (triplicates).

2.5.3. Linearity cream solutions

Portions of base cream of ca. 2.2 g were processed in the same manner as indicated above for cream pre-treatment, and were spiked with appropriate volumes of the miconazole nitrate stock standard solution. Water:acetonitrile mixture was then added to a final volume of 10.0 mL in order to obtain solutions of concentrations which were analogous with those of the linearity standard solutions (triplicates).

2.5.4. Recovery solutions

Laboratory cream samples were prepared by spiking base cream portions with appropriate known amounts of miconazole nitrate standard powder to reach concentrations of 80.0, 100.0 and 120.0% of the expected amount of the analyte in real samples. Triplicates were prepared for each concentration level. The resulting samples were then processed in the same manner as indicated above for cream pre-treatment.

3. Results and discussion

3.1. Method development

3.1.1. Extraction

The sample pre-treatment procedures described in pharmacopoeias for creams containing miconazole nitrate to be assayed by an HPLC method consist in obtaining a suspension of the sample in pure methanol or in a solvent mixture, either by agitation or sonication at room temperature or in a water bath at 40–45 °C. The suspension is then rapidly filtered through a glass microfiber or a membrane filter. This procedure produces a foamy emulsion of the formulation under test, which makes filtration difficult, leading to both low recoveries and low precision. In addition, a late precipitation of excipients in the injector vials was observed, putting the column and equipment safety at risk. On the other hand, the procedure described in the present report (see Section 2), although longer and more laborious than those described in pharmacopoeias, can be straightforwardly implemented. Additionally, it does not need expensive equipment or materials, as those required in the literature methods (i.e. SPE or supercritical extraction fluid) [15,16].

3.1.2. Chromatography

The use of an extra-densely covered column, packed with double end-capping and prepared with an ultra high purity silica support, allowed us to use a pure solvent as the mobile phase, originating both sharp and symmetric peaks. This fact provides the following advantages to the proposed methodology: (a) no buffer solutions are needed, (b) shorter stabilization times are required, and (c) the column lifetime is enlarged.

Several proportions of water:methanol:acetonitrile and flow gradients were evaluated in order to achieve an optimum separation of the analyte from excipients that remain soluble (paraben and degradation products) in a short time of analysis. With the optimized gradient system, the retention time corresponding to miconazole was 8.3 min, being extremely stable among injections. However, the analysis time was set to 15 min, allowing elution of all possible degradation products which could be retained, without the need of a further stabilization time between injections. The wavelength was set at 232 (4) nm, the absorption maximum of miconazole in the elution solvent.

3.2. Method validation

Following ICH recommendations, detection limit and quantitation limit were not evaluated since the developed method is for quantitation of a major component in bulk or in pharmaceutical products.

3.2.1. Selectivity

The selectivity of the method was evaluated injecting by triplicate the following solutions: (a) injection solvent

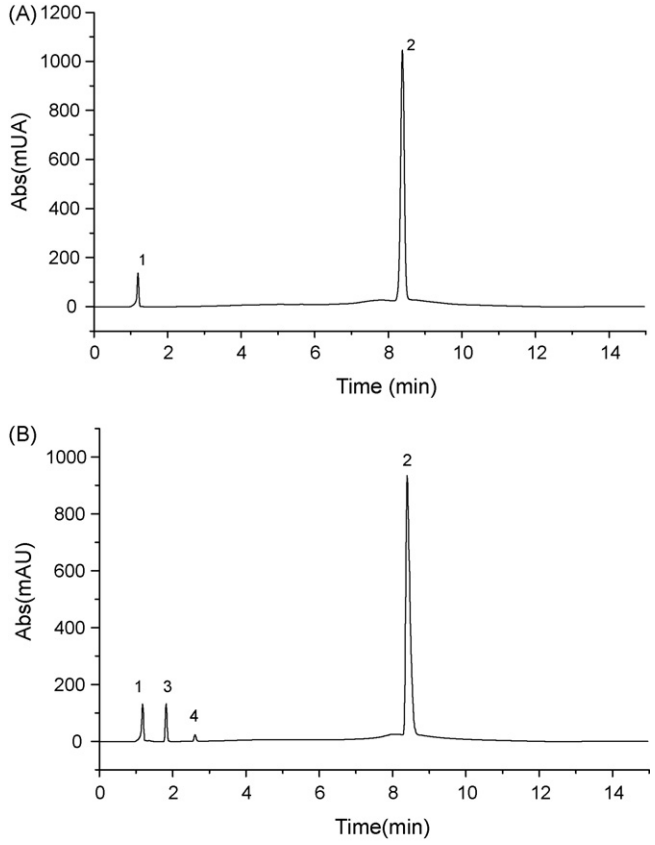

Fig. 2. Typical chromatograms obtained at 232 nm for (A) standard solution of miconazole nitrate 0.53 mg mL^{-1} and (B) miconazole nitrate cream sample showing (1) nitrate ion, (2) miconazole, (3) methylparaben, (4) propylparaben.

(water:metanol:acetonitrile, 30:30:40), (b) a pure standard solution of miconazole nitrate 0.53 mg mL^{-1}, (c) solutions of excipients, (d) solution of matrix, and (e) a cream sample solution. No peaks were observed in solvent chromatograms, whereas two peaks were obtained with the standard solution, corresponding to nitrate ion with retention time (t_r) equal to 1.19 min and miconazole (t_r = 8.38 min). Only methylparaben and propylparaben solutions showed peaks at the selected wavelength. Two peaks were observed in chromatograms for matrix solutions. They were identified by comparing both the retention times and the UV spectra to those for the reference excipient solutions, and corresponded to methylparaben (t_r = 1.80 min) and propylparaben (t_r = 2.60 min). Interestingly, no peaks were observed at t_r values near the miconazole retention time. Finally, when analysing a cream sample solution, the resolution between the analyte and propylparaben peaks was highly satisfactory. Typical chromatograms for standard and sample solutions are shown in Fig. 2.

On the other hand, the peak purity of the analyte was evaluated by the following procedure: (a) recording all spectra in the peak by using a diode array detector, (b) computing the average spectrum, and (c) comparing the difference values between each spectrum and the average with an estimation of the noise threshold. A peak purity factor was calculated as the mean value of all spectra in the peak that are within the threshold [21]. Peak purity factors were 99.95% for miconazole in sample solutions and 99.93% in standard solutions, computed on 135 spectra for each peak. Moreover, spectral matching between analyte peak in standard and sample solutions was of 99.96%. On the basis of these results, it can be concluded that there are no interfering components in the analyte peak.

Table 2
System suitability parameters.

Parameter	Value
Theoretical plates (N)	28,400
Asymmetry (A)	1.117
Tailing (T)	0.922
Capacity factor (K')	3.946
R.S.D. of repeated injections (n = 6) (%)[a]	0.25

[a] Relative standard deviation.

In order to study the degradation of the active ingredient and to get insight into the stability-indicating power of the analytical procedure, ICH recommendations were applied [22]. Three different batches of miconazole nitrate cream, which had previously undergone natural aging during 18 months, were analysed by triplicate, following the above-described procedure. Secondary peaks were also evaluated. A small secondary peak was observed, which was well resolved from the main peaks. Also peak purity of the analyte was maintained, and no interference in the assay was observed.

3.2.2. System suitability

A system suitability evaluation was performed by six standard solution injections, following ICH guidance. Evaluation of analyte peak parameters provided high quality results, as can be appreciated in Table 2. The results agree with those specified in pharmacopoeias and ICH guidelines, demonstrating that the chromatographic system is adequate and reliable.

3.2.3. Solution stability

In order to assure stability of miconazole solution during a period of time considered to be usual in routine analysis, standard and sample solutions were fractionated in four injector vials and left in the injector tray. Each solution was injected every 2 h from a new vial during a total time of 6 h. Percentages of areas respect to the initial area, i.e. t = 0, were calculated and the validity times were established as the time in which the area percentage ranged from 98.0 to 102.0% of the initial value. This study produced the following validity times: 6 h for standard solution and 4 h for sample solution.

3.2.4. Linearity

Linearity was studied for both pure standard and analyte in sample matrix using standard and sample solutions prepared as described in Section 2.5. Peak areas of miconazole were plotted versus concentration, and a least-squares analysis was performed.

For determining the data homoscedasticity, an F-test was employed to compare the extreme variances. The difference between the observed and critical value of F was not significant. The linearity between the response and the concentration over the analyzed range was evaluated using the statistical test suggested by IUPAC [23]. The results obtained showed that the calculated F-values did not exceed the tabulated values.

In order to compare the computed intercepts with the zero value, the confidence intervals were determined. In addition, a t-test was used to compare both the intercept and the slope of regression lines and no significant difference was observed. Results are summarized in Table 3.

3.2.5. Precision

Precision was evaluated at the repeatability and intermediate precision levels. For repeatability analysis, six independent portions of a cream sample were processed through the full analytical method and results were evaluated obtaining an associated R.S.D. (%) value of 0.58.

On the other hand, intermediate precision was evaluated with a new series of six portions of the same sample used in the repeatabil-

Table 3
Statistical analysis for calibration graphs of pure standard and analyte in sample matrix.

Item	Results	
	Standard	Sample matrix
F-Test for homoscedasticity evaluation	4.89 (19)[a]	7.78 (9.12)[b]
F-Test for linearity evaluation	1.13 (4.7)[c]	3.8 (3.8)[d]
t-Test[e] to compare intercepts	0.12 (2.45)	
t-Test[f] to compare slopes	0.02 (2.45)	
Intercept[g]	−29 (76)	27 (122)
Confidence interval for the intercept	[−271; 212]	[−359; 414]

The values between parentheses correspond to critical values at $\alpha = 0.05$ and the following degrees of freedom: [a]($\upsilon_1 = 2$, $\upsilon_2 = 2$), [b]($\upsilon_1 = 4$, $\upsilon_2 = 3$), [c]($\upsilon_1 = 3$, $\upsilon_2 = 6$), [d]($\upsilon_1 = 4$, $\upsilon_2 = 8$), [e]($\upsilon = 6$), [f]($\upsilon = 6$). [g]The values between parentheses correspond to the standard deviation.

ity assay, processed in a different day, 1 week later and by a different analyst. The corresponding R.S.D. (%) was 0.54. A statistical *F*-test was applied to compare the variance with the one computed in the repeatability analysis. The computed *F* value was equal to 1.29, whereas the critical $F_{crit(5,5,\alpha=0.05)}$ is 5.05. Therefore, it can be concluded that no differences exist between the variances obtained. On the other hand, both variances are lower than 2%, indicating the extremely good repeatability of the proposed methodology.

In addition, an R.S.D. (%) value was computed using the mean values of the analysed series; the resulting R.S.D. was 0.90. Owing to the fact that the latter value is lower than 2.8%, the maximum value accepted for AOAC in a recommendation for active ingredients [24], we conclude that the intermediate precision can be considered as excellent.

3.2.6. Accuracy

Solutions prepared as described in Section 2.5 were injected, and the recovery of the known amount of added analyte was computed for each sample. The results obtained are indicative of the good accuracy reached with the proposed methodology, since the mean recovery was included in the interval 100 ± 2%, at each level over the range of 80–120% of the target concentration [24]. Detailed results are shown in Table 4.

In addition, the global recovery, computed for the nine determinations (of the total analytical procedure), was 100.5% (R.S.D.% = 1.70). A statistical Student's *t*-test was applied, which allows us to conclude that no significant difference exists between the recovery obtained and the ideal value 100 at a confidence level of 95%.

3.2.7. Application: assay of commercial cream preparations

Once validated, the developed method was applied to the assay of miconazole nitrate and to the evaluation of degradation products in commercial cream preparations. The obtained results, corresponding to three batches of miconazole nitrate cream, are shown in Table 5. The percentage drug recovery, with respect to the label claimed by the manufacturer, indicates that the active ingredient in samples was present at a level included within the USP requirements of 90.0–110.0 of the labelled amount of miconazole nitrate.

With the aim of performing a further estimation of the potentiality of the developed methodology, three batches, corresponding to aged creams containing miconazole nitrate, gentamicin sulphate and betamethasone 17-valerate were subjected to the whole analytical procedure. As can be observed in Fig. 3, several additional peaks are present, which correspond to betamethasone 17-valerate and both its degradation and isomerization products. Min Li and colleagues demonstrated, through a strategy combining LC–MS with stress studies (forced degradation), that betamethasone 17-valerate follows, at least, two degradation pathway. The isomerization mechanism produces betamethasone 21-valerate, dexamethasone 17-valerate and dexamethasone 21-valerate, since the hydrolytic pathway gives bethametasone and dexamethasone as degradants [25]. By means of DAD employment, we confirmed that all degradants peaks observed in these aged samples chromatograms, showed a high spectral correlation with bethametasone 17-valerate.

Interestingly, miconazole stands perfectly separated from the remaining components. Furthermore, the peak purity is the same as that observed for the standard solution. On the other hand, as can be appreciated in Fig. 3, gentamicin does not interfere in the

Table 4
Recoveries obtained in the accuracy assay.

Level (% of expected concentration)	Recovery (%)	Mean recovery (%)	R.S.D. (%)
80.0	103.2–101.3–101.7	102.2	0.98
100.0	98.5–99.1–100.9	99.5	1.26
120.0	100.9–99.0–99.1	99.7	1.07

Table 5
Results obtained when analysing commercial samples.

Batch	Claimed label (% p/p)	Found[a] (% p/p)	Percentage of claimed label (%)
1	2.00	2.04 (0.03)	102.0
2	2.00	1.98 (0.01)	99.0
3	2.00	1.99 (0.02)	99.5

[a] Average computed for triplicates. Standard deviations are informed between parentheses.

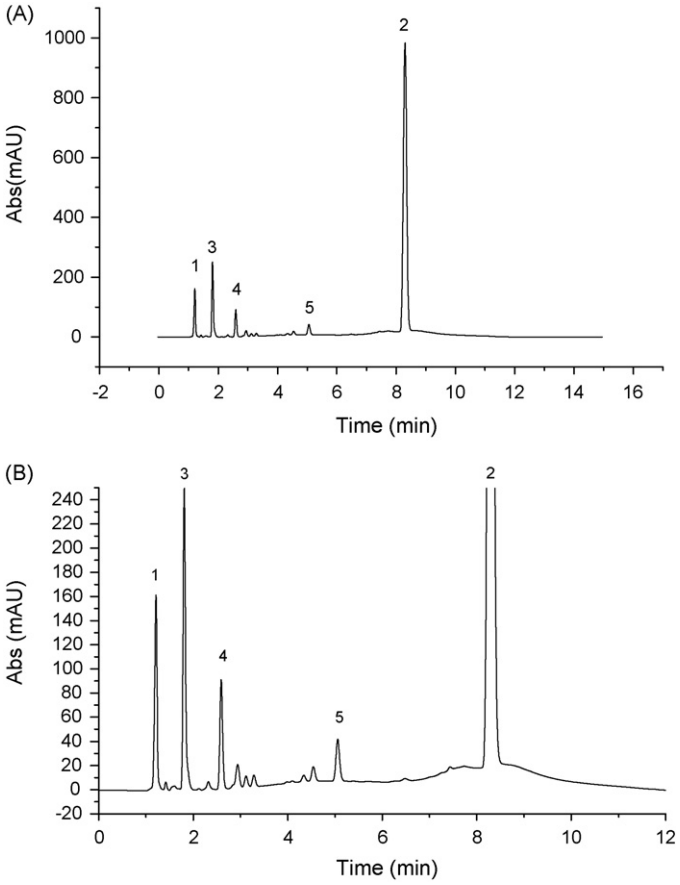

Fig. 3. Chromatogram obtained for a degradated cream sample containing 2.0 g% of miconazole (nitrate), 0.1% of betamethasone (valerate) and 0.1% gentamicine (sulphate). (A) Full scale. (B) Expansion plot in the base line region showing (1) nitrate ion, (2) miconazole, (3) methylparaben, (4) propylparaben, (5) betamethasone and several degradation peaks.

analysis, owing to the fact that the absorbance in the UV region for this compound is negligible.

4. Conclusions

A validated stability-indicating HPLC analytical method for the routine analysis of miconazole nitrate in bulk and cream formulations has been developed. The extractive step of cream samples is simple, requires no sophisticated procedures, and produces excellent analyte recoveries, without the late precipitation of lipophilic compounds occurring in the procedures recommended by pharmacopoeias. The chromatographic method is accurate, precise and specific, and has the ability to separate the analyte from degradation products and excipients which are usually found in cream dosage forms. The analytical procedure has been successfully applied to the determination of miconazole in commercial bulk materials and cream preparations. In addition, the procedure can be applied to the analysis of aged samples to evaluate stability, and to formulations containing other active ingredients.

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