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Short communication

Stability indicating reversed-phase liquid chromatographic determination of metronidazole benzoate and diloxanide furoate as bulk drug and in suspension dosage form

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Abstract

A selective, precise and accurate isocratic RP-HPLC method has been developed and validated for the simultaneous determination of metronidazole benzoate (MB), diloxanide furoate (DF), methyl paraben (MPn) and propyl paraben (PPn) in suspension. The *cis*/*trans* isomerization phenomenon for DF has also been presented. The method uses as stationary phase a Supelco LC-18 DB (15 cm × 4.6 mm) 5 μm column and as a mobile phase a buffer-acetonitrile mixture (70:30, v/v) adjusted to pH of 2.5 at a flow rate of 2.0 mL min^{-1}. The buffer is a 0.005 M KH_2PO_4 solution. The four analytes were well resolved from the degraded solutions peaks. The excepients present in the formulation do not interfere with the assay procedure. The linearity range ($n = 3$) is (0.20130–1.20779 mg mL^{-1}) for MB with R of 0.99985; (0.15790–0.94740 mg mL^{-1}) for DF with R of 0.99987; (0.01131–0.06788 mg mL^{-1}) for MPn with R of 0.99987 and (0.00126–0.00756 mg mL^{-1}) for PPn with R of 0.99991. Precision ($n = 6$) was 0.87% for MB; 1.15% for DF; 1.32% for MPn and 1.27% for PPn. The percentage recoveries ($n = 3$) were 99.1% for MB; 99.6% for DF; 99.1% for MPn and 98.7% for PPn. The proposed method can be utilized for the routine analysis of the four analytes in pharmaceutical dosage form.
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Keywords: HPLC; Metronidazole benzoate; Diloxanide furoate; Method development; Method validation; Isomerization

1. Introduction

The combination of DF and MB (Fig. 1), is useful in the treatment of intestinal and extraintestinal amoebic infections. The drug is indicated in the treatment of acute and chronic intestinal amoebiasis, amoebic hepatitis, amoebic liver abscess, and other systemic infections caused by *E. histolytica* and *giardiasis* [1]. The literature survey reveals several analytical methods for the determination of DF in pharmaceutical dosage form. These methods are titrimetric [2], spectrophotometric [3] and chromatographic [4–6]. Few techniques have been reported for the assay of the DF and metronidazole, not the benzoate, as a combination drug. These methods are classified into packed column super critical fluid chromatography [7], difference spectroscopy [8], and liquid chromatography [9,10].

Since no studies – to the best of our knowledge – have described the determination of DF and MB in pharmaceutical dosage form, this document attempts to describe a new selective, precise, accurate and stability indicating method for the simultaneous determination of DF and MB, including preservatives, in suspension form. Moreover, this paper presents as well, for the first time, the *cis*–*trans* isomerization phenomenon for the DF.

2. Experimental

2.1. Materials

MB pure sample has been obtained from Unique B#MB-041, DF pure sample from Cipex B#E-DF-08, MPn from

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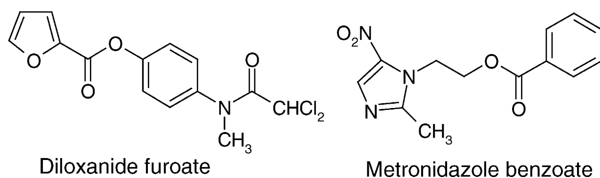


Fig. 1. Chemical structures of MB and DF.

Actico B#M35333 and PPn from NIPA B#8695. Organic solvents for chromatography were of HPLC grade. All reagents and chemicals used were of analytical grade and all were purchased from Sharlou (Spain).

2.2. Instrumentation

The liquid chromatograph consisted of a LC-2010C Shimadzu pump coupled with a UV detector and an autosampler was used for most of the analysis. Data integration was done using Class-VP software for LC peak integration. For the stability indicating study, a Shimadzu SIL-10Advp with photodiode array detector SPD-M10Avp was used. A photodegradation study was carried out using Sun Test XLS+ system with xenon lamp from ATLAS (Germany).

2.3. Chromatography

Chromatography was performed in the reversed-phase (RP) mode. A Supelco LC-18 DB column (15 cm × 4.6 mm) 5 μm (Bellefonte, PA, CA) was used. The mobile phase consisted of (0.005 M) Potassium dihydrogen phosphate–Acetonitrile (70:30, v/v) adjusted with phosphoric acid to a pH of 2.5. The mobile phase was filtered through a 0.45 μm Nylon membrane filter and degassed by sonication. A flow rate of 2.0 mL min^{-1} was maintained. A loop size 20 μl and detection at 260 nm were used.

2.4. Preparation of stock standard solution (level 200%)

A stock standard solution of MB, DF, MPn and PPn, 1.60, 1.24, 0.09 and 0.01 mg mL^{-1}, respectively, was prepared using acetonitrile-deionized water (1:1, v/v) as a solvent.

2.5. Sample preparation

To determine the contents of MB and DF of conventional suspension, label clam: each 5 mL contains 100 mg of metronidazole (as MB 160 mg) and 125 mg of DF. Samples were prepared in Hikma Pharmaceuticals-R&D laboratories. The suspension contains MPn and PPn as preservatives. A sample equivalent to 40 mg of MB and 31.25 mg of DF was transferred from the suspension being examined into a 50 mL volumetric flask with the aid of 25 mL of solvent acetonitrile-deionized water (1:1, v/v). The solution was sonicated for 10 min, and then diluted to volume with same solvent.

2.6. Calibration curves of metronidazole benzoate, diloxanide furoate, methyl paraben and propyl paraben

Appropriate standard solutions were made by diluting the stock standard solution to obtain solutions of concentrations of 25–150% of the sample concentration. Each preparation was chromatographed in triplicate, and a linear regression analysis was performed by plotting concentrations versus peak areas.

2.7. Precision

Method precision was performed by running six composite samples. The average of the six preparations with R.S.D. was calculated.

2.8. Recovery

Placebo sample was prepared and then spiked with MB, DF, MPn and PPn at three levels 25, 100 and 150% of the sample concentration. The mixtures were analyzed by the proposed method ($n = 3$). This was done to check the recovery of the method at different levels of the formulations

2.9. Degradation study

In an attempt to develop a stability-indicating assay method, the samples of placebo, active ingredients and the prepared suspension were subjected separately to the following conditions: heat degradation, acidic degradation using 10 mL of 1N HCl, basic degradation using 10 mL of 1N NaOH and oxidative degradation using 5 mL of 10% H_2O_2. These conditions were all exposed at 85 °C for 2 h. Photodegradation was performed as well for the same samples by exposing them to artificial solar radiation of irradiance of 765 W m^{-2} (irradiance can be defined as the radiant flux incident on a surface per unit area) for 2 h at 25 °C using ATLAS Sun Test. The total irradiance for the UV and Visible region (280–800 nm) is 678.78 W m^{-2} [11]. These solutions, after appropriate dilution with the solvent (neutralization for the acidic and basic degradation), were injected in the chromatographic system.

3. Results and discussion

3.1. Optimization of the LC procedure

The LC procedure was optimized with a view to develop a stability indicating method on one hand. On the other hand, this method should be capable to separate the two preservatives, MPn and PPn, from the drug (Fig. 2A). The mobile phase was adjusted so that the two isomers of diloxanide co-elute and could be applied for the analysis of a bulk drug.

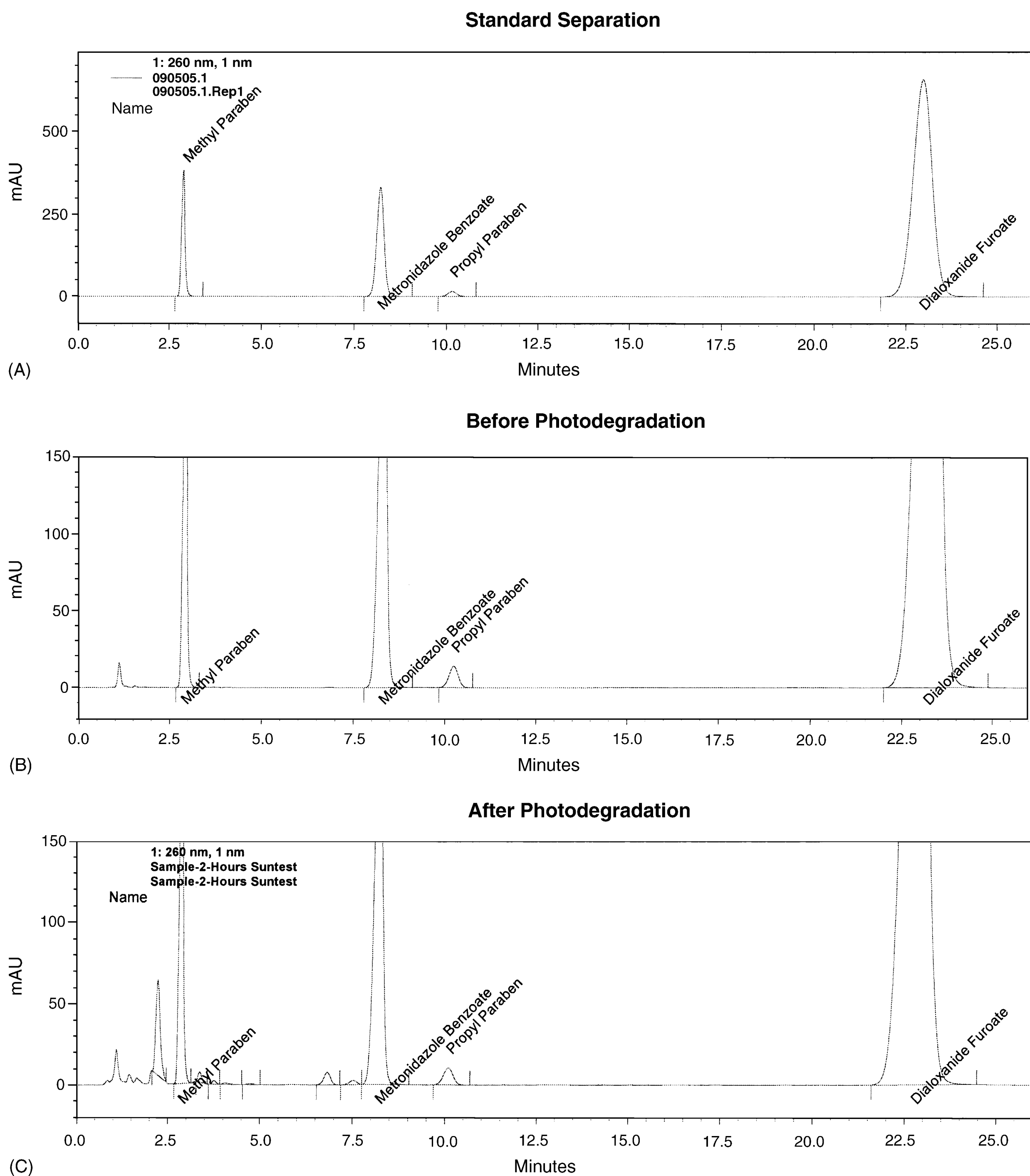


Fig. 2. (A) Typical chromatogram for the standard preparation level 100%; mobile phase: phosphate buffer (0.005 M KH_2PO_4) and acetonitrile (70:30, v/v) pH 2.5; flow rate: 2 mL min^{-1}; (B) finished dosage form before photodegradation and (C) after photodegradation (stressed condition).

Acid-base degradation showed the *p*-hydroxy benzoic acid, due to hydrolysis of the parabens, appearing as a solvent front. The diloxanide was hydrolysed to carboxylated analogues. Additionally, hydrolysis of the metronidazole yielded the benzoic acid, which appeared as a solvent front as well. Using photo diode array detection technique, the placebo and solvent peaks did not show any interference with the active ingredients. All degradants of the suspension for all stressed conditions are well resolved and did not show any interference with MB or DF peaks (Fig. 2B and C).

3.2. *Validation of the method*

3.2.1. *Precision of the assay*

The results in Table 1 showed high precision of the assay method.

Table 1
Precision results

Preparation	MPn (%)	PPn (%)	MB (%)	DF (%)
1	100.3	99.9	100.6	99.7
2	100.7	100.0	99.8	99.0
3	100.8	100.3	99.9	99.6
4	103.1	98.2	100.8	100.9
5	101.8	99.7	100.8	99.4
6	99.2	97.1	98.5	97.4
Average	101.0	99.2	100.0	99.3
R.S.D.%	1.32	1.27	0.87	1.15

Table 2
Recovery studies[a]

Level%		MPn	PPn	MB	DF
25	Average%	99.6	100.4	100.5	100.1
	R.S.D.%	0.8	0.7	0.5	0.8
50	Average%	100.6	99.7	101.4	100.2
	R.S.D.%	0.1	0.2	0.3	0.2
100	Average%	99.1	98.7	99.1	99.6
	R.S.D.%	0.1	0.2	0.3	0.1
150	Average%	98.4	98.6	99.0	100.6
	R.S.D.%	0.1	0.1	0.2	0.1

[a] $n = 3$.

3.2.2. *Recovery of the method*

The proposed method using extraction and subsequent estimation of MB and DF from pharmaceutical dosage form afforded 99–101% as listed in Table 2.

3.2.3. *Calibration curve for the metronidazole benzoate, diloxanide furoate, methyl paraben and propyl paraben*

The calibration curves showed perfect linear relationships over the range 25–150% of the method concentration. The correlation coefficients (R^2) were found to be 0.99985, 0.99987, 0.99987 and 0.99991 for MB, DF, MPn and PPn, respectively (Table 3).

Table 3
Linearity studies

	Concentration ($mg\,mL^{-1}$)			
	MPn	PPn	MB	DF
Level (%)				
150	0.06788	0.00756	1.20779	0.94740
120	0.05431	0.00605	0.96623	0.75792
100	0.04525	0.00504	0.80519	0.63160
80	0.03620	0.00403	0.64416	0.50528
50	0.02263	0.00252	0.40260	0.31580
25	0.01131	0.00126	0.20130	0.15790
Correlation coefficient	0.99987	0.99991	0.99985	0.99987
Slope	5.91E+07	4.99E+07	6.00E+06	4.00E+07
Intercept	30661.5	152.0	57176.0	144913.4

3.3. *Cis–trans isomerization*

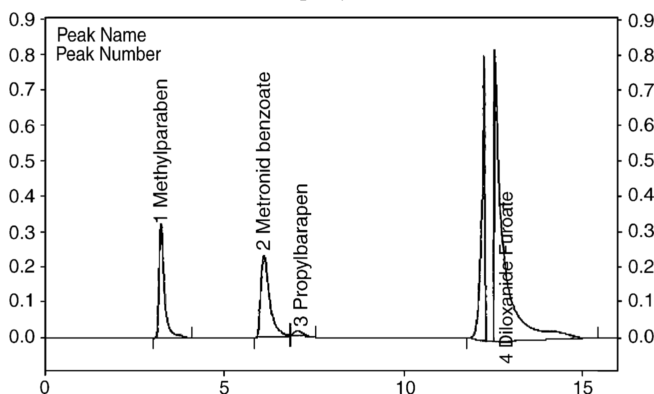
Chromatographic analysis of DF has some peculiarities due to the *cis–trans* phenomenon that was observed for the first time. The *cis–trans* isomerisation of the DF may be attributed to the hindered rotation around the amide bond. Such phenomenon has been observed in HPLC analysis for enalapril and lisinopril [12,13].

3.3.1. *Effect of organic solvent of mobile phase composition*

The influence of the amount of organic modifier on the peak shape and retention time of DF was investigated using different ratios of acetonitrile. A mobile phase composition of phosphate buffer (0.005 M KH_2PO_4) and acetonitrile (70:40, v/v) led to a complete non-baseline separation of the two isomers (Fig. 3). However, a single sharp peak was obtained when the acetonitrile ratio decreased to (70:30). This ratio has been selected as the mobile phase composition for this method.

3.3.2. *Effect of pH*

Chromatograms of DF obtained at two different pHs with a mobile phase composition of phosphate buffer (0.005 M



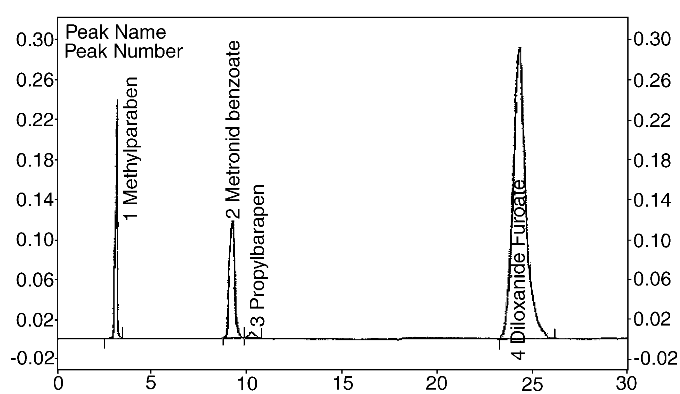


Fig. 3. Effect of the organic modifier on the peak shape and retention time of DF; mobile phase: phosphate buffer (0.005 M KH_2PO_4) and acetonitrile; flow rate: 2 $mL\,min^{-1}$.

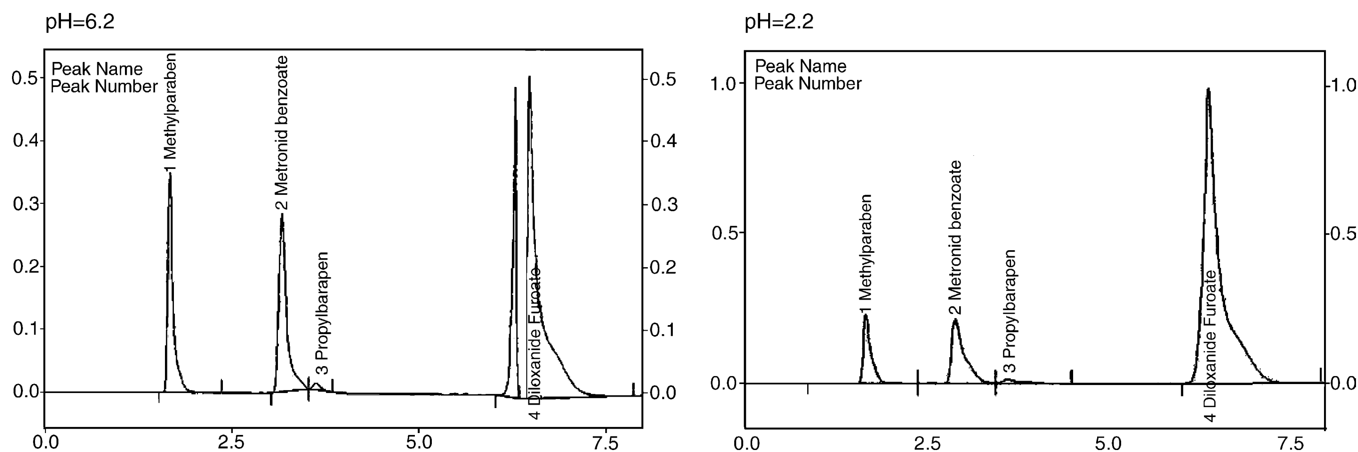


Fig. 4. Effect of the pH on the peak shape and retention time of DF; mobile phase: phosphate buffer (0.005 M KH_2PO_4) and acetonitrile (60:40, v/v); flow rate: 2 mL min^{-1}.

KH_2PO_4) and acetonitrile (60:40, v/v) and a flow rate of 2 mL min^{-1} are illustrated in Fig. 4.

Resolution of the two isomers was achieved at high pH value, pH 6.2, whereas at pH 2.2, a single broad peak has been observed. The peak shape was found to be extremely broad at low pH value. This may be attributed to the interaction between unreacted silanols group of the stationary phase and molecules which are positively charged at low pH.

4. Conclusion

The work has shown that the developed method is precise, accurate, linear and stability indicating. Peak shape and retention time of the DF are affected by operating conditions. However, elution of the DF as a single peak has been achieved by appropriate choosing of mobile phase composition and pH. Application of this method of the analysis of DF and MB in suspension form shows that neither the degradation products nor the excipients, including the parabens preservatives, interfere with the analytical determination. This indicates that the proposed method could be used as a stability indicating method for the determination of DF and MB either in bulk powder or in pharmaceutical formulations.

Acknowledgment

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