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Short communication

A stability-indicating HPLC assay for metronidazole benzoate

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Abstract

A simple and rapid stability-indicating HPLC assay procedure has been developed and validated for metronidazole benzoate. The HPLC conditions were as follows, column: Waters Symmetry C8, 5 μm packing, 4.6 mm × 250 mm; detection: UV at 271 nm; injection volume: 20 μl; mobile phase: acetonitrile—0.1% glacial acetic acid in monobasic potassium phosphate (0.01 M) (40:60, v/v); isocratic elution under ambient temperature at 2.0 ml min^{-1}. The procedure separated metronidazole benzoate and its potential degradation products, metronidazole and benzoic acid, in an overall analysis time of about 6 min with metronidazole benzoate eluting at about 5 min. The injection repeatability was 0.03%, and the intraday and interday repeatability were 0.4 and 0.7%, respectively. The procedure provided a linear response over the concentration range 0.2–800 μg ml^{-1} ($r = 1.0000$) with the limits of detection and quantitation 0.03 and 0.2 μg ml^{-1}, respectively. The solubilities of metronidazole benzoate in water, 0.01 M hydrochloric acid and 0.05 M phosphate buffer, pH 6.8, determined each in triplicate using the procedure, were 0.2 mg ml^{-1} (R.S.D. 7%), 0.4 mg ml^{-1} (R.S.D. 2%) and 0.2 mg ml^{-1} (R.S.D. 8%), respectively. The results show no detectable hydrolysis of metronidazole benzoate in 0.01 M hydrochloric acid at 37 °C or in the mobile phase at ambient temperature in 10 h.

Keywords: Metronidazole benzoate; Metronidazole; Stability; Solubility; Reversed-phase HPLC

1. Introduction

Metronidazole benzoate, a benzoyl ester of metronidazole (Fig. 1), is indicated in the treatment of infections caused by a wide range of anaerobic bacteria, protozoa and bacteroides, including trichomoniasis, amoebiasis, vaginosis and gingivitis [1,2]. The drug manifests its activity against both anaerobic Gram-negative and anaerobic spore-forming Gram-positive bacilli [3]. A suspension of the metronidazole benzoate is often substituted for metronidazole in pediatric oral preparations because of the bland taste of the ester compared to the bitter taste of the free base [4].

Metronidazole benzoate has poor solubility in water, but metronidazole is very soluble (10.5 mg ml^{-1} in water at 25 °C). No significant hydrolysis of the ester was observed in simulated gastric fluid, USP (up to 8 h) and up to 5 h in simulated intestinal fluid, USP [4]. However, sera and urinary excretions of patients administered with metronidazole benzoate show only metronidazole, but no unhydrolyzed metronidazole benzoate [5]. Furthermore, a controlled-release formulation of metronidazole has been developed recently that uses metronidazole benzoate as the source of metronidazole [6]. Thus, to evaluate dissolution of metronidazole benzoate, an analytical procedure capable of quantitating it at low concentrations (due to low solubility in water) in the presence of its degradation products, metronidazole and benzoic acid, is necessary.

Stability-indicating HPLC assay procedures for metronidazole benzoate have been reported [4,7,8]. However, in all the cases, metronidazole (free base), one of the degradation products of metronidazole benzoate, is eluted very early, either with or very close to the solvent front. This renders accurate quantitation of metronidazole difficult. In a stability study of metronidazole benzoate, Matthew et al. [4] used two different HPLC procedures, one for metronidazole benzoate

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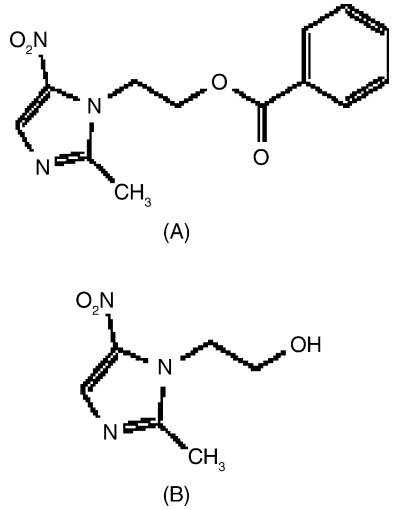

Fig. 1. Structures of (A) metronidazole benzoate and (B) metronidazole.

and the other for its degradation products. In addition, the limit of quantitation (LOQ) of metronidazole benzoate in the previous reports [4,7–9] are too high (20 μg ml^{-1} or higher) for many analytical applications due to its poor aqueous solubility.

In the present study we report a simple, rapid and accurate stability-indicating HPLC assay procedure that can quantitate metronidazole benzoate and its degradation products simultaneously. Our results show that the lower limit of the linear range of the procedure is 0.2 μg ml^{-1} (LOQ), two orders of magnitude lower than that reported previously.

2. Experimental

2.1. Materials

Metronidazole benzoate was obtained from a local pharmacy. The USP Metronidazole RS was obtained internally. Monobasic potassium phosphate, hydrochloric acid and sodium hydroxide solution (50%, w/w) were from Fisher Scientific (Fair Lawn, NJ). Acetonitrile was obtained from Allied Signal Inc., Burdick & Jackson (Muskegon, MI) and Benzoic acid, USP grade, was from Mallinckrodt Specialty Chemicals Co. (Paris, KY). All chemicals are of analytical grade and used as received.

2.2. HPLC

The HPLC system consisted of a Hewlett-Packard 1100 series pump, a solvent degasser, an autosampler, a variable wavelength detector and ChemStation software Version A.04.01 (Hewlett-Packard, Avondale, PA). A Waters Symmetry C8 column, 5 μm, 4.6 mm × 250 mm was used. The separation was carried out under isocratic elution with acetonitrile—0.1% glacial acetic acid in monobasic potassium phosphate (0.01 M) solution (40:60, v/v) at a flow rate of 2.0 ml min^{-1} with UV detection at 271 nm. The column temperature was ambient and an injection volume of 20 μl was used.

The standard solution was prepared immediately before use. A 1 mg ml^{-1} solution of metronidazole benzoate was prepared in acetonitrile, which was diluted with the mobile phase to give a 0.1 mg ml^{-1} solution.

2.3. Method validation

Method validation was performed as per USP 27-NF22 [10]. The following validation parameters were addressed: specificity, precision, linearity, limits of detection and quantitation, and stability of metronidazole benzoate in the mobile phase.

2.4. Solubility

The HPLC procedure was used to determine the solubility of metronidazole benzoate. The solubilities of metronidazole benzoate in water, 0.01 M hydrochloric acid and 0.05 M phosphate buffers, pH 6.8, were determined by adding the drug to the solvent in excess of the expected solubility and equilibrating in a water bath at 37 °C with a shaker for 10 h. A portion of the clear supernatant was carefully withdrawn, diluted 10-fold with the mobile phase and analyzed using the HPLC procedure. The solubility was also determined with the samples warmed to 50 °C and then equilibrated at 37 °C for 10 h and samples equilibrated at 37 °C for 24 h. The solubility in each medium was determined in triplicate.

3. Results and discussion

3.1. HPLC procedure development

Metronidazole (free base) is eluted either with or very close to the solvent front in all the cases when we used previously reported stability-indicating HPLC assay procedures [4,7,8]. This makes detection of metronidazole, one of the degradation products, ambiguous and its accurate quantitation difficult. In addition, the procedure reported by Matthew et al. [4] gave significant fronting of the metronidazole benzoate peak (tailing factor <1, calculated as per Ref. [11]). Our efforts to modify the procedure by changing the concentrations of acetonitrile and acetic acid in the eluent and varying the column temperature did not improve the results.

The previous procedures used C18 columns [4,7,8]. When we changed to a C8 column to reduce hydrophobicity of the column to increase retention of protonated metronidazole (under the elution condition), it eluted after the solvent, permitting us to quantitate metronidazole benzoate, metronidazole and benzoic acid in the same run with an overall analysis time of about 6 min (Fig. 2A). In addition, the C8 column shows good peak shape (tailing factor as defined by

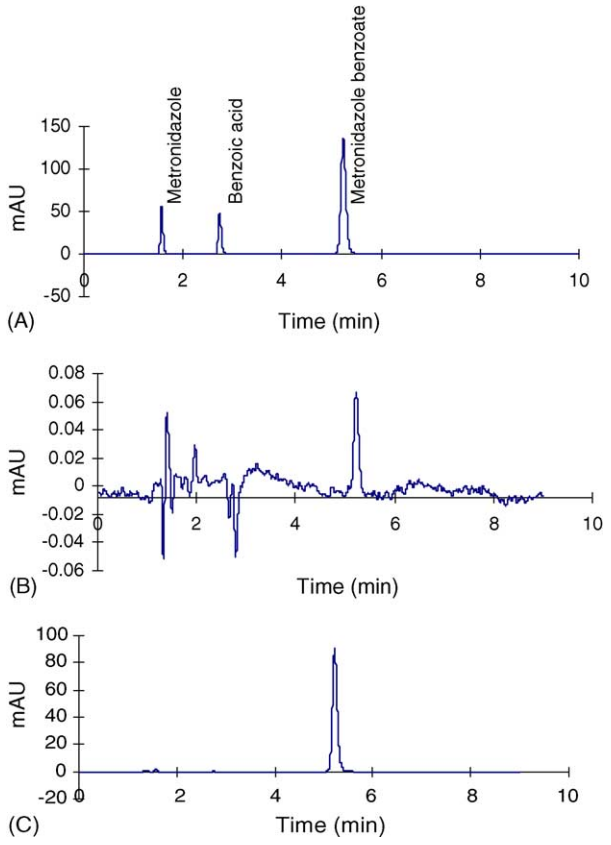

Fig. 2. Chromatograms of: (A) a solution containing 0.08 mg ml^{-1} metronidazole benzoate, 0.01 mg ml^{-1} metronidazole and 0.01 mg ml^{-1} benzoic acid in mobile phase; (B) a 0.03 μg ml^{-1} solution (LOD) of metronidazole benzoate; (C) a solution of metronidazole benzoate obtained by incubating the drug in 0.01 M hydrochloric acid at 37 °C for 10 h. Chromatographic conditions, Column: Waters Symmetry C8, 5 μm packing, 4.6 mm × 250 mm. Mobile phase: acetonitrile—0.1% glacial acetic acid in 0.01 M monobasic potassium phosphate (40:60, v/v) at a flow rate of 2.0 ml min^{-1}. Detection: UV, 271 nm. Injection volume: 20 μl.

the USP-NF [11] was 1.1). The resolution between metronidazole benzoate and benzoic acid (closest peak) peaks was 7 or greater, and the number of the theoretical plates (efficiency) [11] was 8600 (Table 1).

3.2. Validation

3.2.1. Specificity

The peak area responses of the metronidazole benzoate were compared between solutions in mobile phase containing 0.08 mg ml^{-1} metronidazole benzoate and 0.08 mg ml^{-1} metronidazole benzoate containing 0.01 mg ml^{-1} each of metronidazole and benzoic acid. The chromatogram from the latter solution is shown in Fig. 2A. The peak area responses and the retention times of metronidazole benzoate from the two solutions were not significantly different at 95% confidence limit. Fig. 2A also shows that the peaks of metronidazole, benzoic acid and metronidazole benzoate are well resolved with approximate retention times of 1.6, 2.7 and 5.3 min, respectively. Thus, the presence of potential degradation products in the solution did not interfere with the determination of metronidazole benzoate under our HPLC conditions.

3.2.2. Precision

The injection (system) precision was determined by performing six replicate injections of the standard solution (nominally, 0.1 mg ml^{-1}). The procedure precision (intraday repeatability [10]) was determined by performing five replicate assays of independently prepared samples of metronidazole benzoate. The R.S.D. values were 0.03 and 0.4%, respectively (Table 1). The assay results of metronidazole benzoate solutions (nominally, 0.1 mg ml^{-1}), prepared and assayed on each of three consecutive days averaged 100.2% and the precision (R.S.D.) was 0.7%.

3.2.3. Limit of quantitation, limit of detection and linearity

It was expected that the procedure would be used to determine very low concentrations of metronidazole benzoate due to its low aqueous solubility. Hence, the limit of detection (LOD) and LOQ were evaluated. The LOD and LOQ were taken as the concentrations of the analyte that gave responses 3 and 10 times that of the background noise, respectively. The baseline noise was determined by measuring the peak-to-peak noise in a 30 s section of the baseline immediately after the metronidazole benzoate peak is completely eluted. Samples containing metronidazole in the concentration range 0.01–4 μg ml^{-1} were injected, five replicates each, and the LOD and LOQ were calculated from the results. The LOQ was determined to be 0.2 μg ml^{-1} and five replicate injections of the drug prepared at this concentration gave an R.S.D. of 1.1% (Table 1). Fig. 2B shows a typical chromatogram of metronidazole benzoate at 0.03 μg ml^{-1} (LOD). The LOQ was found to be 0.03 μg ml^{-1}.

The procedure showed a linear response over a wide range of concentrations, 0.2–800 μg ml^{-1} (r = 1.0000) (Table 1). The intercept of the line was not significantly different from 0 (95% confidence limit).

The LOQ values reported previously for the HPLC assay procedures for metronidazole benzoate are 20 μg ml^{-1} or higher [4,7–9]. Our preliminary results suggest that these values are too high to obtain a reliable dissolution profile of the ester in water due to the poor aqueous solubility of the drug (see later). However, our results show a two orders of magnitude lower LOQ than what was reported before. The low LOQ permits use of the current procedure in dissolution studies.

3.2.4. Stability of metronidazole benzoate in the mobile phase

The stability of the metronidazole benzoate solution in the mobile phase (standard solution) was investigated by making injections of the solution over a period of 10 h at room temperature. The chromatogram showed no peak corresponding to the elution times for metronidazole and benzoic acid (data not shown). Also, there was no significant change in the peak

Table 1
Summary of the performance parameters of the HPLC procedure for metronidazole benzoate

Parameter	Value	Comments
Efficiency (theoretical plates)	8600	Standard preparation (0.1 mg ml^{-1})
Tailing factor (USP)	1.1 (R.S.D. = 4%, n = 6)	Measured at width of peak at 5% of peak height at 0.1 mg ml^{-1}
Linearity		
Range	0.2–800 μg ml^{-1}	Intercept was not significantly different from zero (95% CL)
Correlation coefficient	1.0000	
Equation		
Intercept	7 (S.E. = 3)	S.E. = standard error
Slope	15385 (S.E. = 8)	
Precision (n: number of injections or samples)		
Injection repeatability	0.03%	n = 6
Intraday repeatability	0.4%	n = 5
Interday repeatability	0.7%	n = 3
Limit of quantitation (LOQ)		
Concentration	0.2 μg ml^{-1}	
Precision at LOQ	1.1%	
Limit of detection (LOD)	0.03 μg ml^{-1}	
Solution stability	≥10 h	0.1 mg ml^{-1} solution of metronidazole benzoate in mobile phase was stable for at least 10 h

area response of metronidazole benzoate, indicating that a solution of metronidazole benzoate is stable in the mobile phase for at least 10 h at room temperature.

3.3. Solubility and solution stability

The HPLC procedure was used to determine the solubilities of metronidazole benzoate in water, 0.01 M hydrochloric acid and 0.05 M phosphate buffer, pH 6.8. After equilibrating for 10 h at 37 °C, the solubility of metronidazole benzoate was 0.2 mg ml^{-1} (R.S.D. 7%) in water, 0.4 mg ml^{-1} (R.S.D. 2%) in 0.01 M hydrochloric acid and 0.2 mg ml^{-1} (R.S.D. 8%) in 0.05 M phosphate buffer, pH 6.8. Neither warming the solutions to 50 °C before equilibration to facilitate dissolution nor using longer equilibration times up to 24 h significantly changed the solubility of metronidazole benzoate in these solvents. We did not find any report of solubility of metronidazole benzoate in these media in the literature. However, the results show that the solubility of the ester at 37 °C is about 50 times less than the reported solubility of the free base at 20 °C [12].

Fig. 2C shows the metronidazole benzoate peak, but none for either metronidazole or benzoic acid when a diluted solution obtained by equilibrating metronidazole benzoate in 0.01 M HCl at 37 °C was injected. Similar results were obtained when a 10-fold concentrated solution was injected. The results indicate no significant hydrolysis of metronidazole benzoate in 0.01 M HCl at 37 °C in 10 h. Based on a similar observation, Matthew et al. [4] concluded that the practice of substituting metronidazole benzoate for metronidazole in the treatment of gastrointestinal infections might be inappropriate. However, Alestig et al. showed that sera and urinary excretions of patients administered with metronidazole benzoate show only metronidazole, but no unhydrolyzed metronidazole benzoate [5]. Thus, it appears that the in vivo hydrolysis of metronidazole benzoate may involve a mechanism other than the simple acid–base hydrolysis.

4. Conclusions

A stability-indicating HPLC assay was developed for the quantitation of metronidazole benzoate and its potential degradation products, metronidazole and benzoic acid. The procedure is specific, accurate, rapid, precise and sensitive with an LOQ of 0.2 μg ml^{-1}, and shows linearity over a wide concentration range. The procedure permitted an accurate determination of solubility of the drug. The solubilities of metronidazole benzoate in water, 0.01 M hydrochloric acid or 0.05 M phosphate buffer, pH 6.8, at 37 °C were measured using the HPLC procedure.

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