Quantitation of Metronidazole in Pharmaceutical Suspension Using High Performance Liquid Chromatographic Method

Saeed-ul-Hassan, Shafique Hussain and Muhammad Tayyab Ansari*

1University College of Pharmacy, Punjab University, Lahore, Pakistan
2Department of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan

Abstract.- A simple and rapid HPLC (High performance liquid chromatography) assay procedure has been developed and validated for the quantitative determination of metronidazole benzoate for raw material and commercially available suspensions. The HPLC conditions used were, column: C18, (0.5µm particle size, 150×4.6mm); detection: UV at 254 nm; injection volume: 20 µl; mobile phase: methanol and de-ionized water (64:40, v/v); isocratic elution under ambient temperature at flow rate of 1.0 ml min⁻¹. The procedure separated metronidazole benzoate, in an overall analysis time of about 10 minutes with metronidazole benzoate eluting at about 7.7 minutes. The injection repeatability was 0.05%, the intraday and interday repeatability were 0.59 and 0.91%, respectively. The procedure provided a linear response over the concentration range 10–1000 µg ml⁻¹ (r² = 1.0000) with the limits of detection and quantitation 0.0158 and 10 µg ml⁻¹, respectively. The results show no detectable hydrolysis of metronidazole benzoate in 10 hours.

Key words: Metronidazole benzoate, Reverse-phase HPLC, suspension, methanol, precision

INTRODUCTION

Metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) belongs to nitroimidazole group that is a relatively inexpensive, highly versatile drug with clinical efficacy against broad spectrum anaerobic bacteria. The uses of metronidazole for antiprotozoal therapy have been reviewed extensively (Freeman et al., 1997; Johnson, 1993; Stanley, 2003; Nash, 2001). It is clinically effective in trichomoniasis, amebiasis, and giardiasis, as well as in a variety of infections caused by obligate anaerobic bacteria, including Bacteroides, Clostridium, Fusobacterium, Peptococcus, Peptostreptococcus, Eubacterium, and microaerophilic bacteria such as Helicobacter and Campylobacter spp. Metronidazole antimicrobial properties are thought to derive from the formation of toxic-free radicals by intracellular reduction.

Both parenteral and enteral routes have been used to administer metronidazole. Nevertheless, the need for additional dosage forms have been warranted for patients who require a different dosage form than what is commercially available (e.g., patients who are incapable of swallowing tablets or capsules or pediatric patients who are incapable of swallowing available dosage forms). Recently a number of oral suspensions of metronidazole benzoate have been marketed.

For estimation of metronidazole, several methods have been used i.e., spectrophotometry e.g. metronidazole and hycanthone (Bahia and Moussa, 1982), metronidazole and nalidixic acid (Parimoo et al., 1996), metronidazole and diloxanide furoate (Indravadan et al., 1997); thin layer chromatography; Supercritical fluid chromatography e.g. metronidazole and nalidixic acid (Viddesh et al., 1998), electrochemistry, and voltammetry e.g. metronidazole (Ozkan et al., 1998), high-performance liquid chromatography (HPLC) e.g. reverse phase HPLC has been used for the simultaneous determination of metronidazole and miconazole in pharmaceutical dosage forms. Chromatography was carried out on a C18 reversed-phase column, using a mixture of methanol–water (40+60, v/v) as mobile phase, at a flow rate of 1.0 ml min⁻¹. Sulfamethoxazole was used as an internal standard and detection was performed using a diode array detector at 254 nm. The method produced linear responses in the concentration ranges 10–70 and 1–20 µg ml⁻¹ with detection limits 0.33 and 0.27 µg ml⁻¹ for metronidazole and miconazole, respectively. This procedure was found to be convenient and reproducible for analysis of these
Similarly simultaneous determination of metronidazole benzoate (MB), diloxanide furoate (DF), methyl paraben (MPn) and propyl paraben (PPn) in suspension was studied (Mishal and Sober, 2005) in which mobile phase, a phosphate buffer-acetonitrile mixture (70:30, v/v) adjusted to pH of 2.5 at a flow rate of 2.0 mL min\(^{-1}\). In addition simultaneous estimation of amoxicillin and metronidazole at single wavelength (254 nm) using C\(_{18}\) column with buffered mobile phase (pH 4.0) and was not affected by excipients (Naser et al., 2007). Metronidazole benzoate was studied by Daniel et al. (2005) who used UV at 271 nm; injection volume: 20 µl; mobile phase, acetonitrile and 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 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. To our knowledge very few reports are available for metronidazole benzoate and these are not related to suspension.

In the present study we report a simple, rapid and accurate HPLC assay procedure that can quantitate metronidazole benzoate in raw material as well as in suspension and this method is unaffected by excipients present in suspension. Mean recovery percentage of metronidazole benzoate by this procedure is higher than previously reported.

## MATERIALS AND METHODS

### Chemicals

Metronidazole Benzoate Standard (Kindly provided by Sanofi-Aventis Pakistan Limited, Karachi), Metronidazole Benzoate Raw material (Kindly provided by Sanofi-Aventis Pakistan Limited, Karachi), HPLC Grade Methanol (Merck Darmstadt, Germany), De-ionized Water, Metronidazole Suspension: Flagyl 200mg/5ml (Aventis Ltd Karachi), Phosphoric Acid (Merck Darmstadt, Germany).

### Preparation of standard solutions

Metronidazole benzoate (643 mg) reference standard equivalent to 400 mg of metronidazole was weighed accurately on an analytical balance and was taken in a 200 ml volumetric flask. It was dissolved completely with methanol and the volume was made up to the mark with the same solvent. Standard solutions of different concentration i.e., 50, 100, 200, 500 and 1000 µg ml\(^{-1}\) were prepared. These solutions were filtered through 0.45 micron membrane filter. Standard graph was plotted concentration against area under curve by High performance liquid chromatography (HPLC).

### Preparation of sample solutions

Five ml of the metronidazole suspension was taken in 200 ml volumetric flask with the help of pipette. Added 150 ml of methanol and dissolved completely by ultrasonic bath. The volume was made up to the mark with the same solvent. 2.5 ml of the above solution was taken in another 100 ml volumetric flask, added de-ionized water up to the mark and mixed well. Filtered through 0.45 micron membrane filter. The concentration of the above solution is 50 µg ml\(^{-1}\).

### Preparation of mobile phase for HPLC

Methanol (HPLC grade) and de-ionized water were mixed in the ratio of 650:400 ml and the pH was adjusted to 2.5 by phosphoric Acid. The mobile phase was filtered through 0.45 µ membrane filter by vacuum filtration unit and then degassed for 15 minutes by ultrasonic bath.

### HPLC analytical procedure

An aliquot of 20 µl sample was injected on HPLC (Shimadzu LC-10ATvp) column (C18, 0.5µm particle size, 150×4.6mm) having Flow Rate 1 ml/minute at ambient temperature using detector (UV 254 nm; chart speed 5 mm/minute; attenuation 8 (adjustable) and sensitivity 0.01). Samples were used in five replicate. Major peak response of metronidazole benzoate in assay preparation and standard preparation were recorded in the chromatogram.

### Validation of analytical method

The analytical method for measurement of
the drug was validated for specificity, linearity (evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares), accuracy (assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range, 3 concentrations 3 replicates each of the total analytical procedure reported as percent recovery by the assay of known added amount of analyte in the sample), precision (assessed by a minimum of 6 determinations at 100 percent of the test concentration) and sensitivity.

**Calculations**

The percentage of metronidazole benzoate was calculated by the formula given below.

\[
\% \text{ age of metronidazole benzoate as per labeled amount} = \frac{\text{SPA}}{\text{STA}} \times \frac{C_2}{C_1} \times \text{F}
\]

where SPA is the peak area of the metronidazole benzoate in assay preparation, STA is the peak area of the metronidazole benzoate in standard preparation, \(C_2\) is the concentration in \(\mu\text{gml}^{-1}\) of metronidazole benzoate in standard preparation, \(C_1\) is the concentration in \(\mu\text{gml}^{-1}\) of metronidazole benzoate in assay preparation and \(\text{F}\) is the exact percentage purity of the metronidazole benzoate reference standard.

**RESULTS AND DISCUSSION**

**HPLC procedure development using metronidazole raw material**

After the preparation of standard curve (Fig.1) for metronidazole benzoate (MB) reference material, raw material was analyzed by the proposed method at different concentrations and compared, one of the comparisons is shown in Figure 2. In order to identify the impurities under isocratic conditions, the mixtures of methanol with water in different combinations were assayed as the mobile phase using C18 packing as stationary phase. Binary mixture of methanol-/water in proportion of 65:40 (v/v), proved to be better than the mixture of acetonitrile-water for the separation since the chromatographic peaks were better defined and resolved, and almost free from tailing. Among several flow rates tested (0.5-2 ml min\(^{-1}\)), the rate of 1.0 ml min\(^{-1}\) was the best with respect to location and resolution of analytical peaks.

**Fig. 1. Standard graph between metronidazole concentration (\(\mu\text{g/ml}\)) on X-axis While chromatographic area (mV) on Y-axis**

**Fig 2. Chromatograms showing peak of Metronidazole as metronidazole benzoate in standard solution containing 200 \(\mu\text{g ml}^{-1}\) (A) and its raw material containing 250\(\mu\text{g ml}^{-1}\) (B) using mobile phase, methanol-water (65:40; v/v), flow rate 1.0 ml min\(^{-1}\) and detection wavelength 254 nm.**

Retention time of metronidazole in standard solution was 7.751±0.00025 (0.016) and in raw material 7.755±0.00089 (0.029) which indicate consistency of retention time using proposed mobile phase ratios and from the Table I it is clear that the percentage purities of the raw material of metronidazole benzoate at different concentrations are within the BP range of (99-101%).
Table I.- Characteristics of linear regression analysis of metronidazole benzoate (MB)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg ml⁻¹)</td>
<td>10-1000</td>
<td>Intercept was not significantly different from zero (96% CL)</td>
</tr>
<tr>
<td>Slope</td>
<td>12248</td>
<td>Area under curve (mV)</td>
</tr>
<tr>
<td>Intercept</td>
<td>25106</td>
<td>Area under curve (mV)</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Injection repeatability</td>
<td>0.05%</td>
<td>n=6</td>
</tr>
<tr>
<td>Intraday repeatability</td>
<td>0.59%</td>
<td>n=4</td>
</tr>
<tr>
<td>Interday repeatability</td>
<td>0.91%</td>
<td>n=4</td>
</tr>
<tr>
<td>Solution stability</td>
<td>≥ 10 hrs</td>
<td>Standard solution of metronidazole benzoate in mobile phase was stable for at least 10 hPrecision 1.2%</td>
</tr>
<tr>
<td>Limit of quantitation (LOQ)</td>
<td>10 µg ml⁻¹</td>
<td></td>
</tr>
<tr>
<td>Limit of detection (LOD)</td>
<td>0.0158 µg ml⁻¹</td>
<td></td>
</tr>
<tr>
<td>Mean Recovery % in Standard reference</td>
<td>100.01</td>
<td></td>
</tr>
<tr>
<td>Mean Recovery % in Raw material</td>
<td>100.09</td>
<td></td>
</tr>
</tbody>
</table>

* Mean values represent three different sample standards for each concentration.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of preparation</th>
<th>Composition (Each 5ml contains)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flagyl suspension</td>
<td>Metronidazole benzoate eq. to metronidazole base =200mg</td>
<td>Sanofi-Aventis Limited, Karachi, Pakistan</td>
</tr>
<tr>
<td>2</td>
<td>Diazol suspension</td>
<td>Metronidazole benzoate eq. to metronidazole base =200mg</td>
<td>Atco Limited, Karachi, Pakistan</td>
</tr>
<tr>
<td>3</td>
<td>Menidazole suspension</td>
<td>Metronidazole benzoate eq. to metronidazole base =200mg</td>
<td>Werrick Limited, Islamabad, Pakistan</td>
</tr>
<tr>
<td>4</td>
<td>Metrozil suspension</td>
<td>Metronidazole benzoate eq. to metronidazole base =200mg</td>
<td>Siza International Limited, Lahore, Pakistan</td>
</tr>
<tr>
<td>5</td>
<td>Metrozine suspension</td>
<td>Metronidazole benzoate eq. to metronidazole base =200mg</td>
<td>Searle Limited, Karachi, Pakistan</td>
</tr>
<tr>
<td>6</td>
<td>Protomet suspension</td>
<td>Metronidazole benzoate eq. to metronidazole base =200mg</td>
<td>Geofman Limited, Karachi, Pakistan</td>
</tr>
</tbody>
</table>

The injection (system) precision was determined by performing six replicate injections of the standard solution (nominally, 200 µg ml⁻¹). The characteristics of regression equations and the working concentrations are given in Table I. The limit of detection (LOD) and limit of quantitation (LOQ) of the procedure are also shown in Table II, which were calculated according to the 3s /m and 10s /m criterions, respectively, where s, is the standard deviation of the peak area ratios (four injections) of the sample and m is the slope of the corresponding calibration curve (Swartz and Krull, 1997). The intra- and inter-day variations of the method were determined on commercial products using three replicate injections of four different concentrations, which were prepared and analysed on the same day and on three different days over a period of two weeks, respectively. The R.S.D. values were 0.59 and 0.91%, respectively (Table II). The assay results of metronidazole benzoate in standard solutions (nominally, 200 µg ml⁻¹), prepared and assayed on each of three consecutive days averaged 97.69% and the precision (R.S.D.) was 0.91%. This indicates a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs. Sample solutions analysed after 48 h did not show any appreciable change in assay values.

Table II.- Names and dosage form of commercially used suspensions of metronidazole benzoate.

Assay in pharmaceutical dosage forms

On the basis of above results, the proposed method was applied for the determination of metronidazole benzoate in commercial suspensions
DETERMINATION OF METRONIDAZOLE BY HP LC METHOD

Fig. 3. HPLC Chromatograms of Flagyl MB suspension (A), Diazol (B), Menidazole (C), Metrozil (D), Metrozine (E) and Protomet suspension (F)

(Table II) which comprised various excipients [sucrose, ethanol, special magnesium silico aluminate (veegum), sodium saccharine dihydrate, sodium dihydrogen phosphate dihydrate, methylparahydroxybenzoate, concentrate lemon essence, deterpenated orange essence, propylpara-hydroxybenzoate]. Figure 2 shows typical chromatograms obtained for the analysis of MB in suspensions. The differences between the amount claimed and those measured were very low and the RSD values were within the acceptable windows mentioned by pharmacopoeias. When various quantities of suspensions (Equivalent to 400 µg ml\(^{-1}\) of metronidazole benzoate) were assayed by HPLC, the mean values of Flagyl suspension was 409.80, Diazol suspension (405.60 µg ml\(^{-1}\)), Menidazole (415.00 µg ml\(^{-1}\)), Metrozil suspension (402.80 µg ml\(^{-1}\)), Metrozine suspension (410.92 µg ml\(^{-1}\)), Protomet suspension (408.80 µg ml\(^{-1}\)).

The recovery of the procedure was carried out by spiking the already analyzed standard samples of metronidazole benzoate (MB). Percentage recovery by Flagyl suspension was 102.27, Diazol 101.40, Menidazole 103.75, Metrozil suspension 100.70,
Metrozine suspension 102.73 and Protomet suspension 102.20 respectively (n=6), indicating that the results are accurate, precise and there is no interference from the common excipients used in the suspension formulation.

Accuracy was assessed using Flagyl in minimum of nine determinations over not less than three concentration levels (60, 250 and 700 µg ml\(^{-1}\) covering the specified range (3 concentrations/3 replicates each of the total analytical procedure). Statistical analysis showed concentration 100.37±1.08 (1.04) that the precision (R.S.D.) was 1.71%.

CONCLUSIONS

It can be concluded that the proposed method is sufficiently sensitive and reproducible in the analysis of metronidazole benzoate in raw material and in pharmaceutical suspension forms within a short analysis time (<8 min). The proposed HPLC method was validated by evaluation of the validation parameters e.g. linearity range, solution stability, relative standard deviation of slope and intercept, correlation coefficient, within- and between-day reproducibility, LOD, LOQ values, for this technique were obtained.

ACKNOWLEDGEMENT

Authors are thankful to Dosaco Laboratories, Lahore who provided laboratory facilities to complete this work.

REFERENCES


(Received 24 June 2010, revised 18 March 2011)