International Scholars Journals

African Journal of Agriculture ISSN 2375-1134 Vol. 11 (7), pp. 001-004, July, 2024. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Review

Cost-Effective Liquid Chromatography Technique for Analyzing Metronidazole in Biological Fluids

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Accepted 3 February, 2024

A rapid and cost effective method for the analysis of metronidazole in biological samples was developed. The extraction method is a simple single-step liquid-liquid process that has eliminated the need for costly extraction and evaporation equipment. The mobile phase consists largely of water, making the method cheap to run with less than 6 min total analytical time per sample. The calibration curve was linear from 0 to 2.00 µg/ml. The regression coefficient was 0.99. The method is highly sensitive, with limit of detection of 1 ng/ml. The coefficient of variation for within-day run was less than 4% while that of day-to-day run was less than 6%. There were no interfering peaks from endogenous materials in the serum. The method was validated and used for pharmacokinetic studies.

Key words: Metronidazole, Liquid chromatographic, biological fluids.

INTRODUCTION

Metronidazole is a 5-nitronimidazole derivative with activity against anaerobic protozoa and anaerobic bacteria. The drug is readily absorbed following oral administration. Metronidazole is widely distributed; it appears in most body tissues and fluids including bile, bone, breast milk, cerebral abscesses, saliva, and generally achieves concentrations similar to those in plasma.

The choice of an analytical method is usually governed by the intrinsic analytical properties of the drug molecule or its amenability to chemical derivatisation to render it compatible to quantitation. In addition, such factors as sample size, sample environment, detection method and even the method for quantitation must be taken into account, since these affect the reliability of the quantitation. Various chromatography methods have been reported for the analysis of metronidazole in pharmaceutical dosage form and biological fluids (Akay et al., 2002; Yeung et al., 1998). Currently, reversed phase and ion-pair high–performance liquid chromatography with UV detection represent the analytical method of choice for the quantitative determination of metronidazole in human plasma, urine and/or saliva (Jessa et al., 1996; Yeung et al., 1998; Galmier et al., 1998). The older methods used for the analysis of metronidazole required expensive reagents and elaborate extraction procedure. The present study aims to provide simple, sensitive and rapid but cost- effective HPLC method of assaying metronidazole both in pharmaceutical and biological samples.

EXPERIMENTAL

Reagents and solutions

All the reagents used are analytical grades. Stock solutions (1 mg/ml) of metronidazole and sulphamethoxazole (Reference Standards) were prepared in 0.1 N HCl. Each stock solution was protected from light, stored at 4ºC in the refrigerator and freshly diluted for analysis when needed. Metronidazole USP reference standard was obtained from Rockville, USA, while sulphamethoxazole was obtained from Sigma Aldrich, USA and Hydrochloric acid Analar from BDH Chemical Ltd., Poole England. The HPLC grade methanol was obtained from Sigma Aldrich.

Chromatographic conditions

The chromatograms were from an Agilent 1100 series quartenary LC pump system, equipped with ultraviolet detector. Wavelength for detection was 276 nm. The stationary phase was a silica-based

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Table 1. Within day and day-to-day precision for serum metronidazole determination**.**

Parameter	Concentration (µg/ml)	% Coefficient of variation	N
Within- day run	0.10	3.2	5
	1.00	1.6	5
	2.00	1.2	5
Day-to-day	0.10	5.5	5
	1.00	1.7	5
	2.00	1.0	5

Table 2. Percentage recoveries of metronidazole from different concentrations of rat serum.

Concentration (µg/ml)	% Recovery
0.100	92.48
0.200	97.17
0.500	99.43
1.000	99.66
2.000	99.89
MEAN	97.73
± SD	3.19

ultrasphere C-18 column (5 mm, 2.0 mm x 25 cm). The mobile phase consisted of methanol: water in a ratio of 25:75. The mobile phase was pumped through the column at ambient temperature and at a flow rate of 1 ml/min.

Sample collection

Blood samples were collected into clean sample bottles from adult Wister rats for the calibration curves. Samples were then collected at time 0, 0.5, 1, 2, 3, 6 and 8 h interval after oral administration of metronidazole 7.5 mg/kg for the measurement of serum concentrations. All samples were centrifuged at 2000 g for 10 min and the serum was stored frozen at -20ºC until analyzed.

Serum extraction

Into 10- ml extraction tubes were placed 0.2 ml of serum spiked with 200 ng sulphamethoxazole (I.S.). The mixture was made alkaline with 0.5 ml of 2 M NaOH and 2 ml of dichloromethane was added. The resulting mixture was then vortex mixed for 1 min. The tubes were centrifuged at 3000 g for 10 min and the organic layer was then transferred to fresh tubes and evaporated to dryness on the water bath at temperature 40ºC. The dried residue was reconstituted with 100 µl of 0.1 N HCl. 20 µl aliquot was injected onto the HPLC system.

Percentage recovery

0.2 ml of blank rat serum was spiked with metronidazole standard solution to give predetermined concentrations. To each sample was added 200 ng of sulphamethoxazole (I.S.) and the resulting solution was taken through the extraction procedure previously described under serum extraction. The absolute recovery was determined by comparing the peak area ratios of extracted metronidazole standard with those obtained by direct injections of the same concentrations of the drugs.

Calibration curve

Calibration curves, which were based on peak-area ratio to the concentration of the drug, were prepared by spiking drug-free serum with a standard metronidazole (1 mg/ml) to give a concentration range of 0-2.00 µg/ml and same quantity of sulphamethoxazole (200 ng) was added. The level of metronidazole in an unknown sample was derived from these values.

Precision of the method

0.2 ml of blank serum was spiked with metronidazole standard solution to give predetermined concentrations. To each sample was added 200 ng of internal standard and the resulting solution was taken through the extraction procedure previously described. The precision was estimated by computing the peak area ratio and the coefficient of variation (CV) of the three different concentrations for five samples at each level was studied per each set of analysis and average determined.

Lower limit of quantitation

Limit of detection of the drug was checked from the direct injection of diluted samples from the stock solution.

RESULT AND DISCUSSION

The method is highly sensitive, with the lower limit of quantitation of metronidazole at 1 ng/ml. The coefficient of variation for within- day was less than 4% while that of day-to-day-run was less than 6% (Table 1) and the extraction efficiency was high with percentage recoveries >98% (Table 2). The calibration curve was linear in serum with a regression of r=0.9998 as shown in Figure 1.

The resultant chromatograms from the system HPLC with metronidazole and I.S showed that the HPLC method developed is selective as it provided a good resolution of metronidazole from the internal standard and there was no interference from endogenous materials. The retention times of metronidazole and sulphamethoxazole (I.S) were 4.5 and 3.2 min respectively (Figures 2 and 3). The single extraction step using a small volume of serum (0.2 ml) with less than 6 min total analytical time per sample is appropriate for a routine assay procedure. This allows for the analysis of about 12 samples per h. It is noteworthy that no metabolite was detected in this study, which is in agreement with other published studies (Menelaou et al., 1999; Akay

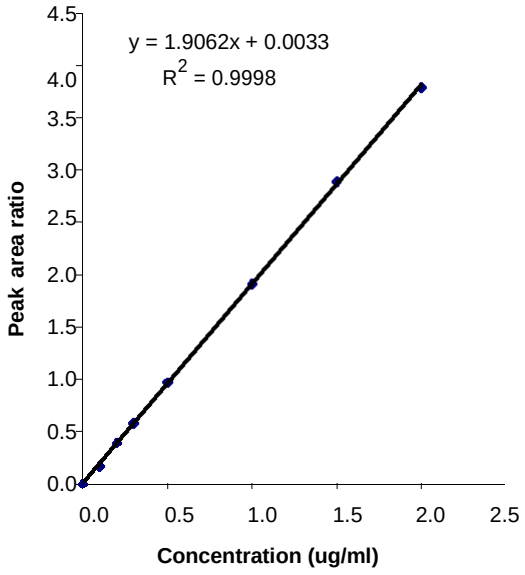

Figure 1. Calibration curve for metronidazole in rat serum using HPLC.

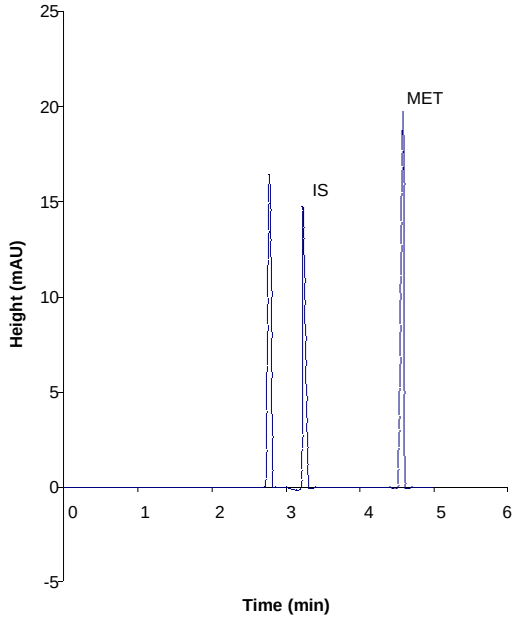

Figure 2. Chromatogram of blank rat serum spiked with metronidazole (2 g/ml) and sulphamethoxazole (200 ng) using solvent system methanol: water (25:75).

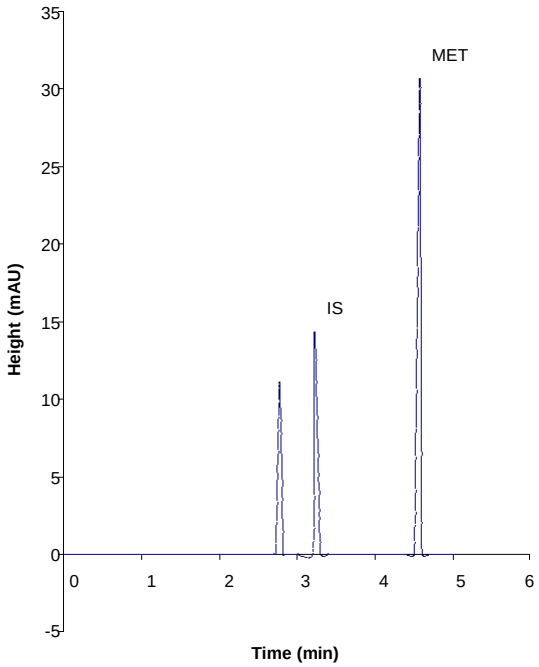

Figure 3. Chromatogram of an extract of serum obtained from rat 1 h after oral administration of 7.5 mg/kg metronidazole. IS = Internal standard, Met = metronidazole.

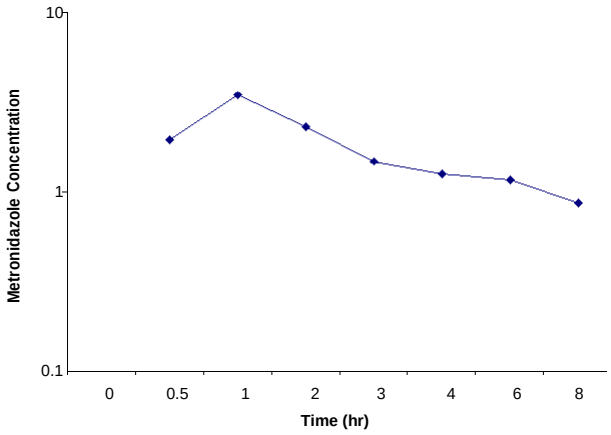

Figure 4. Serum concentration-time curve of metronidazole in rats following oral administration of 7.5 mg/kg metronidazole.

et al., 2002; Klimowicz et al., 2002).

Despite the fact that many modern HPLC methods have been described for the assay of metronidazole (Klimowicz et al., 2002; Wibawa et al., 2001; Menelaou et al., 1999), there is justification for investigating newer methods, especially where they are more cost-effective and there is an appreciable reduction in the procedural time compared to older methods. The use of expensive evaporating equipment is circumvented since very small quantity of organic solvent was used. It may be applied to both research and therapeutic drug monitoring as demonstrated by the concentration time curves (pharmacokinetic curve in Figure 4).

In conclusion, the HPLC assay method developed in this study using a reversed-phase system was found to be simple, cost-effective and sensitive for assaying metronidazole concentration as low as 1 ng/ml in serum.

ACKNOWLEDGEMENT

We acknowledge the National Institute for Pharmaceutical Research and Development, Idu-Abuja, Nigeria, for funding this work.

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