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Development of a HPLC Method for the Estimation of Metronidazole in Human Plasma

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Varying amount of metronidazole (200 to 5000 ng) and fixed quantity (120 ng) of nevirapine (internal standard) was added to the blank human plasma (0.5 mL), and extracted with diethyl ether. The mixture was centrifuged and diethyl ether layer separated, dried and reconstituted with mobile phase. Fifty microlitres of this solution were injected into a reverse phase C-18 column using a mobile phase consisting of acetonitrile and 0.01 M potassium dihydrogen phosphate buffer (pH adjusted to 3.0 with 5% orthophosphoric acid) in the ratio of 20 : 80% v/v and the eluents were monitored at 318 nm. The method was validated for its linearity, precision and accuracy. The method is simple, precise, specific, less time consuming and accurate for the estimation of metronidazole in plasma samples.

Key Words: Metronidazole, Plasma, Reverse phase liquid chromatography.

INTRODUCTION

Metronidazole, chemically, is 1-(hydroxyethyl)-2-methyl-5-nitroimidazole and active against a wide variety of anaerobic protozoal parasites and anaerobic bacteria. Metronidazole is clinically effective in trichomoniasis, amoebiasis and giardiasis as well as in a variety of infections caused by anaerobic bacteria, including bacteroides, clostridium and helicobacter species[1]. Several analytical methods have been reported for the estimation of metronidazole by HPLC in plasma[2–5], gastric fluids[2], urine[3], feces[4] and milk[5]. In the present study a sensitive, accurate and precise HPLC method has been developed for the estimation of metronidazole in human plasma.

EXPERIMENTAL

Metronidazole (purity 99.2 to 100.4%; USP) and nevirapine (purity 99.5 to 99.9%) were obtained from M/s Alkem Laboratories Ltd., Mumbai, India and M/s Vimta Labs Ltd., Hyderabad, India respectively. Acetonitrile, methanol and water were HPLC grade and were obtained from M/s Qualigens Fine Chemicals, Mumbai, India.

A gradient high pressure liquid chromatography (Shimadzu HPLC Class VP series) with two LC-10AT VP pumps, variabie wavelength programmable UV/Vis Detector SPD-10A VP, CTO-10AS VP column oven (Shimadzu), SCL-10A VP system controller (Shimadzu) and RP C-18 column (250 mm × 4.6 mm I.D., particle size 5 μm; YMC, Inc., Wilmington, NC 28403, USA) was used. The HPLC system was equipped with the software "Class-VP series version 5.03 (Shimadzu)".

HPLC conditions: The mobile phase components acetonitrile and 0.01 M potassium dihydrogen phosphate buffer (pH adjusted to 3.0 with orthophosphoric acid) were filtered through 0.2 μm membrane filter before use and were pumped from the solvent reservoir at a ratio of 20 : 80 % v/v to the column at a flow rate of 1 mL/min. The volume of each injection was 50 μL.

Calibration solutions: Six sets of plasma samples with varying drug concentrations were prepared by spiking drug-free plasma with an appropriate volume (100 μL) of a known amount of metronidazole at a concentration range of 200 to 5000 ng/0.5 mL of plasma along with 120 ng/0.5 mL of nevirapine (internal standard) solution.

Plasma extraction procedure: A 0.5 mL aliquot of plasma was accurately measured into a 10 mL glass tube with a teflon-lined cap, followed by addition of 100 μL of 120 ng/0.5 mL of nevirapine (internal standard) solution. The mixture was vortexed to ensure complete mixing of contents. Three millilitres of diethyl ether were added, shaken well for 10 min and centrifuged for 10 min at 4,000 rpm. The organic layer (2.5 mL) was carefully transferred to glass tubes and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 300 μL of mobile phase and injected to the HPLC column. The eluents were detected using UV detector at 318 nm. The peak areas of metronidazole and intemal standard were recorded. The ratio of peak area of metronidazole to that of internal standard (nevirapine) was calculated and the regression of the peak area ratio against the plasma concentration of the drug was also calculated using the least squares method of analysis.

Precision: Aliquots of blank plasma (0.5 mL) were spiked with 100 μL of internal standard solution (containing 120 ng) and of metronidazole solutions (100 μL) with various strengths to yield concentrations of 300, 500 and 1000 ng/0.5 mL of plasma. Each sample was extracted as described above, and injected into the HPLC column (n = 5). Each sample was prepared in triplicate on three consecutive days, and injected in to the HPLC column (n = 5) to observe the precision of the method.

Accuracy: The preanalyzed plasma samples containing 300, 500 and 1000 ng/0.5 mL were added with known quantity of metronidazole (1000 ng) and subjected to HPLC analysis, in triplicate, as described above. The difference in the measured concentration and that of the added quantity (1000 ng) was expressed in per cent recovery.

RESULTS AND DISCUSSION

The run time of the method was set at 10 min. Metronidazole and nevirapine (internal standard) appeared on the chromatogram in about 3.8 min and 9.00 min respectively (Fig. 1). When the same drug solution was injected 5 times, the retention time of the drug and internal standard were same. Table-1 shows the mean peak area ratios of metronidazole to that of internal standard solutions for 5 such determinations. When the concentration of metronidazole and its respective peak area ratios were subjected to regression analysis by least squares method, a high correlation coefficient was observed ($r = 0.9999 \pm 0.0012$) in the range of 300 to 5000 ng/0.5 mL only. However, the minimum quantifiable concentration was found to be 200 ng/0.5 mL of human plasma. The regression of metronidazole concentration over its peak area ratio was found to be $Y = 0.05215 + 0.00026X$ where 'Y' is the peak area ratio and 'X' is the concentration of metronidazole. This regression equation was to estimate metronidazole in plasma or in validation study (precision and accuracy).

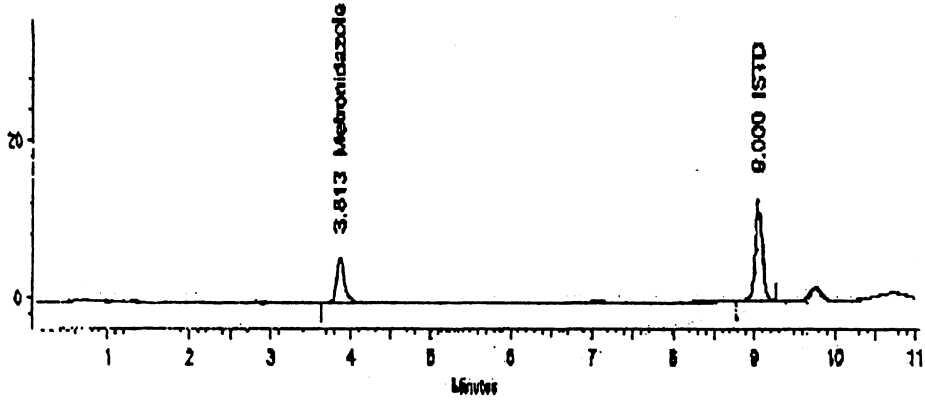

Fig. 1. Typical chromatogram of metronidazole in human plasma

TABLE 1
CALIBRATION CURVE FOR THE ESTIMATION OF METRONIDAZOLE IN HUMAN PLASMA BY HPLC METHOD

Amount of metronidazole added to 0.5 mL human plasma (ng)	Peak area ratio*	C.V. (%)
0	0	0.00
200	0.0852	0.85
300	0.1329	0.84
500	0.1854	1.20
1000	0.3166	0.98
2000	0.5789	0.82
5000	1.3659	0.79

*Mean of five determinations
Regression equation: $Y = 0.5215 + 0.00026X$ ($r = 0.9999$)

The present HPLC method was also validated for intra- and inter-day variation. The coefficient of variation (CV) in the peak area ratio for five replicate injections was found to be less than 1.2%. Also, the inter-day variation (3 days and five injections) was found to be less than 1.8%. Thus, the results show that this HPLC method is highly reproducible. When a known amount of drug solution (1000 ng) was added to preanalysed plasma samples (300, 500 and 1000 ng/0.5 mL), there was a high recovery (mean recovery 101.27%) of metronidazole indicating that this HPLC method is highly accurate. Hence the proposed HPLC method was found to be simple, precise, highly accurate, specific and less time consuming than the reported methods[2-5].

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