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Note

Sensitive high-performance liquid chromatographic method for the determination of metronidazole and metabolites

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Metronidazole (Flagyl, Clont; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is effective in the treatment of anaerobic infections [1]. Metronidazole is also gaining wider use in the treatment of Crohn's disease, a debilitating enteritis [2].

Previous methods for the determination of metronidazole and its metabolites in biological fluids have utilized bioassays [3], gas—liquid chromatography (GLC) [4], polarography [5], thin-layer chromatography (TLC) [6, 7] and high-performance liquid chromatography (HPLC) [7—10]. Bioassays and polarography are nonspecific and time-consuming. GLC involves the organic extraction of metronidazole, with subsequent derivatization, and does not include metabolites. The HPLC assays described do not incorporate an internal standard and can be contaminated by protein precipitates leading to poor resolution and shorter column life.

The HPLC method described here is rapid, involving no organic extraction, utilizes an internal standard to minimize day-to-day variability, does not have problems with protein precipitation, and simultaneously measures metronidazole and its major metabolites (of which hydroxymetronidazole is biologically active) in biological fluids.

MATERIALS AND METHODS

Chemicals

Metronidazole, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (hydroxymetronidazole), and 1-acetic acid-2-methyl-5-nitroimidazole (metronidazole acetic acid) were kindly provided by Bayer AG, Leverkusen, F.R.G. Tinida-

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zole, used as an internal standard, was provided by Pfizer GmbH, Karlsruhe, F.R.G. All other chemicals were of HPLC grade or better.

High-performance liquid chromatograph

A Waters (Milford, MA, U.S.A.) high-pressure liquid chromatography system was used for all determinations. This system included a Model M6000-A high-pressure pump, a Model 450 variable-wavelength UV detector, and a Wisp 710 A automatic injector. A Hewlett Packard (Avondale, PA, U.S.A.) Model 3380A integrator was used for data processing. The carrier was a solution of 0.005 *M* KH_2PO_4 (pH 4.5)—methanol—tetrahydrofuran (82.6:16.5:0.9) pumped at a flow-rate of 1.4 ml/min through a reversed-phase μC_{18} Bondapak column (30 cm × 3.9 mm; average particle size 10 μm; Waters Associates), with the effluent monitored at 324 nm.

Sample preparation

To a 1.5-ml conical microfuge tube (Eppendorf GmbH, Hamburg, F.R.G.) were added 200 μl of plasma or urine (urine being previously diluted 1:10 with distilled water), 50 μl of ethanol containing 10 mg per 100 ml tinidazole, and 50 μl of 0.1 *M* $ZnSO_4$. The tubes were capped, vortexed for 15 sec, and placed in a refrigerator for 15 min. The tubes were then centrifuged for 2 min in an Eppendorf microfuge. An aliquot (10 μl) of the superantant was injected into the liquid chromatograph.

For the measurement of the glucuronide conjugates of metronidazole and hydroxymetronidazole in urine, 1 ml of urine was mixed with 4 ml of acetate buffer (0.2 *M*, pH 4.5). To this mixture were added 50 μl of glucuronidase—sulfatase (10,000 Fishman units/ml; Sigma GmbH, Munich, F.R.G.). This solution was incubated at 37°C for 16 h. Following incubation, 1 ml of the incubate was mixed with 1 ml of distilled water. A 0.2-ml aliquot of this mixture was prepared as described above for the plasma samples.

RESULTS AND DISCUSSION

With the system described, the retention times for metronidazole acetic acid, hydroxymetronidazole, metronidazole, and tinidazole (internal standard) were 2.4, 3.0, 4.7, and 6.7 min, respectively (Fig. 1). Interfering peaks from pooled plasma, urine, and urine samples prepared for glucuronide analysis were not observed. As seen in Fig. 1, no peak tailing was found, a problem reported earlier [9], enabling the use of peak height or peak area in the calculation of standard curves. The calibration graph was linear over a concentration range of 1—40 μg/ml when plasma and urine were spiked with the substances measured. The limit of detection was 0.05 μg/ml for metronidazole and metabolites. The accuracy of the method was tested by adding the compounds of interest to plasma and urine and extracting ten samples. The percentage coefficient of variation (C.V.) of extracted plasma samples at a concentration of 2 μg/ml metronidazole, hydroxymetronidazole, and metronidazole acetic acid was 4.1, 4.3 and 4.3%, respectively. From urine, the C.V. values were 5.1, 5.9, and 4.4%, respectively. Day-to-day variability was tested by extracting frozen plasma and urine samples previously spiked with metronidazole and metab-

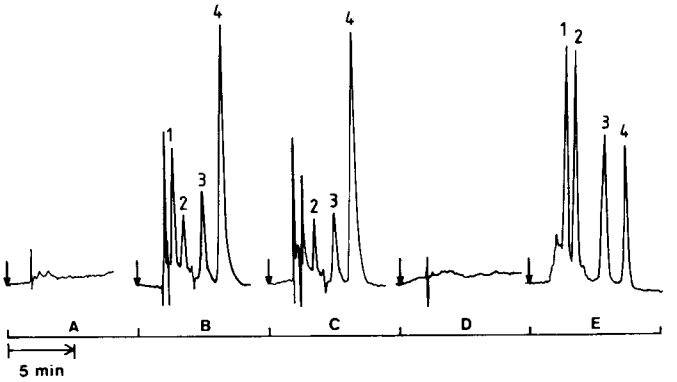

Fig. 1. HPLC chromatograms from: (A) extract of blank plasma; (B) extract of blank plasma to which metronidazole (2 μg/ml) and metabolites (2 μg/ml each) and internal standard (5 μg) had been added; (C) extract of plasma obtained from a patient receiving metronidazole; (D) extract of blank urine; (E) extract of urine obtained from a patient receiving metronidazole. The peaks indicated are (↓) injection point; (1) metronidazole acetic acid; (2) hydroxymetronidazole; (3) metronidazole; (4) tinidazole (internal standard).

olites over a three-week period. The C.V. of plasma samples was 5.6% for metronidazole, 4.5% for hydroxymetronidazole, and 3.5% for the acid metabolite. Similar results were found for the extraction of urine samples.

This method has been used to study the pharmacokinetics of metronidazole following intravenous or oral single dosing (Table I) and multiple dosing [11]. The method is accurate, as indicated by the low C.V. of extracted samples, and enables the simultaneous measurement of metronidazole and major metabolites in plasma and urine. Bioassays have proved to be inaccurate due to the antimicrobial action of hydroxymetronidazole [12] which is also found in plasma. Polarography suffers from the same problem, due to the reduction of all nitroimidazoles present, metronidazole and its metabolites, which measures the total amount of nitroimidazole present, but does not differentiate between metronidazole and its metabolites [5]. The described method utilizes no organic extraction, as do the GLC and TLC methods [4, 6, 7] and one of the HPLC methods [10].

TABLE I

URINARY EXCRETION OF METRONIDAZOLE AND ITS METABOLITES IN TWO PATIENTS FOLLOWING A SINGLE 400-mg ORAL DOSE OF METRONIDAZOLE

Excretion is expressed as percentage of dose eliminated in 48 h.

Compound	Patient I	Patient II
Metronidazole (free)	9.5	2.5
Metronidazole (conjugated)	6.1	3.6
Metronidazole 1-acetic acid	26.9	10.2
Hydroxymetronidazole (free)	38.1	8.5
Hydroxymetronidazole (conjugated)	9.9	12.2

The previously described HPLC methods used no internal standard, the use of which increases accuracy [8, 9, 13]. In addition, protein precipitation was reported to be a problem if samples were left standing for long periods of time, as would be the case when automatic injectors are used [9]. The use of $ZnSO_4$ and cooling described here has eliminated this problem, with no additional precipitation being seen over 6 h. Also, by not using perchloric acid [8] or trichloroacetic acid for protein precipitation, column life is greatly extended.

In conclusion, the described method is rapid and can be used for plasma and urine without carrier modification. Metronidazole is assayed simultaneously with its major metabolites in plasma and urine [11], with an increased accuracy due to the use of an internal standard.

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