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Note

Rapid determination of metronidazole in human serum and urine using a normal-phase high-performance liquid chromatographic column with aqueous solvents

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Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is effective against various infectious diseases caused by both aerobic and anaerobic micro-organisms and sensitizes hypoxic cells to radiation damage. A large number of reversed-phase high-performance liquid chromatographic (HPLC) methods have been described to analyze metronidazole in body fluids [1-12]. The procedure reported is a normal-phase system with 2 min retention for metronidazole with a simple sample preparation.

MATERIALS AND METHODS

Reagents and materials

Metronidazole was supplied by the United States Pharmacopeia (U.S.P.). Ammonium phosphate and acetonitrile were HPLC grade, and the water used was doubly distilled. Human serum and urine samples were obtained from human volunteers and frozen at -20°C. Within a week of storage, each sample was thawed at room temperature and analyzed.

Standard solutions were prepared by accurately weighing approximately 50 mg of U.S.P. reference standard metronidazole and dissolving in 500 ml of mobile phase. This stock solution was accurately diluted with mobile phase 1:1, 1:10, 1:20, 1:50, 1:100 and 1:1000 for a wide range of concentrations used both for obtaining standard curves and needed for validation studies. These solutions were stored in glass at 0°C until needed.

Apparatus

Analyses were performed on a modular instrument with in-line solvent

and precolumn filters of approximately 0.45 μm porosity. A saturator column containing 37- μm silica was located between the pump (Beckman-Altex 110A) and the autoinjector, which was either a Perkin-Elmer Model 420B or 600. Either a Kratos Model 773, a Perkin-Elmer Model 85 or Beckman 165 detector set to 320 nm was used. A laboratory automation system (Hewlett-Packard Model 3356) was used to process the data. A Kipp and Zonen two-pen recorder was also used to plot chromatograms as back-up to the computer.

Chromatographic conditions

Analytical columns successfully used were Chromegabond Diol, 5 and 10 μm particle size, 30 cm \times 4.6 mm (E.S. Industries, Marlton, NJ, U.S.A.) and LiChrosorb Diol, 10 μm particle size, 25 cm \times 4.6 mm (E. Merck, Darmstadt, F.R.G.). Mobile phase consisted of 0.005 *M* ammonium phosphate, adjusted to pH 6.5 with potassium hydroxide solution—acetonitrile (40:60) flow-rate 1 ml/min. Absorbance range adjustments are unnecessary with the computer system.

Sample preparation

Serum samples were thawed at room temperature. Afterwards, 1.0 ml from each sample tube was diluted with 1.0 ml of pH 6.0 buffer (1.65 g of dipotassium hydrogen phosphate and 98.35 g potassium dihydrogen phosphate dissolved in 1 l of distilled water) and transferred into a properly treated (cones soaked in distilled water for at least 1 h) Centriflo[®] membrane cone (Type CF50A).

The serum and phosphate buffer were centrifuged at 900 *g* for 15 min. An aliquot from the supernatant was mixed with an equal portion of mobile phase and injected. Urine samples were handled in a similar manner with 2.0 ml of urine mixed with 2.0 ml of phosphate buffer in the above mentioned membrane cone. Standards were dissolved in phosphate buffer, added to serum at 10 $\mu\text{g}/\text{ml}$ or to urine at 100 $\mu\text{g}/\text{ml}$ and were prepared with the appropriate clinical samples.

Procedure

Standard and sample solutions are injected in the sequence: standard₁, standard₁, standard₂, sample₁, sample_n, standard₁ or ₂, where *n* is number of samples and depends on the stability of the system. The first injection was rejected since it is the most susceptible to column anomalies. Responses of the two standards should be within 1.5%. Failure to achieve reasonable agreement requires reinjection, reweighing and preparation of the standard, reequilibration, or repair of the HPLC system. Purity of one standard should be calculated (as if the injection was an unknown) using integrators or computers. Obtaining the designated purity value verifies the system and arithmetic program. Quantitation of purity can be based on area or height.

Drug administration

Healthy male volunteers were administered metronidazole by 30-min intravenous infusions in a single dose, 500 mg. Metronidazole was purchased as a 1 mg/ml solution in isotonic citrate and phosphate-buffered saline. Administra-

tion was by intravenous infusion using a syringe pump (Harvard Apparatus Compact Infusion Pump).

RESULTS AND DISCUSSION

A rapid assay for metronidazole in human serum and urine has been developed using a normal-phase diol HPLC column with a reversed-phase type of mobile phase. Such a system is chromatographically stable with no deterioration even after 500 injections.

The sample preparation of serum in previously described methods required solvent pretreatment, centrifugation and addition of an internal standard for quantification [3,5,7,9,11]. The sample pretreatment in this method simply requires ultrafiltration and mixing of the supernatant with mobile phase. Internal standards were not required since recoveries obtained were consistent and near theory.

Chromatograms of human serum prior to dosing, at the end of infusion and 15 min after the end of infusion clearly show absence of any interferences (see Fig. 1). Fig. 2 shows a computer-enhanced chromatogram of control urine showing the absence of interference with metronidazole and Fig. 3 a typical chromatogram of drug in urine collected 0 to 2 h after dosing. Peak widths are exaggerated due to the time scale.

After 24 h, urinary excretion of metronidazole reached 22% of administered

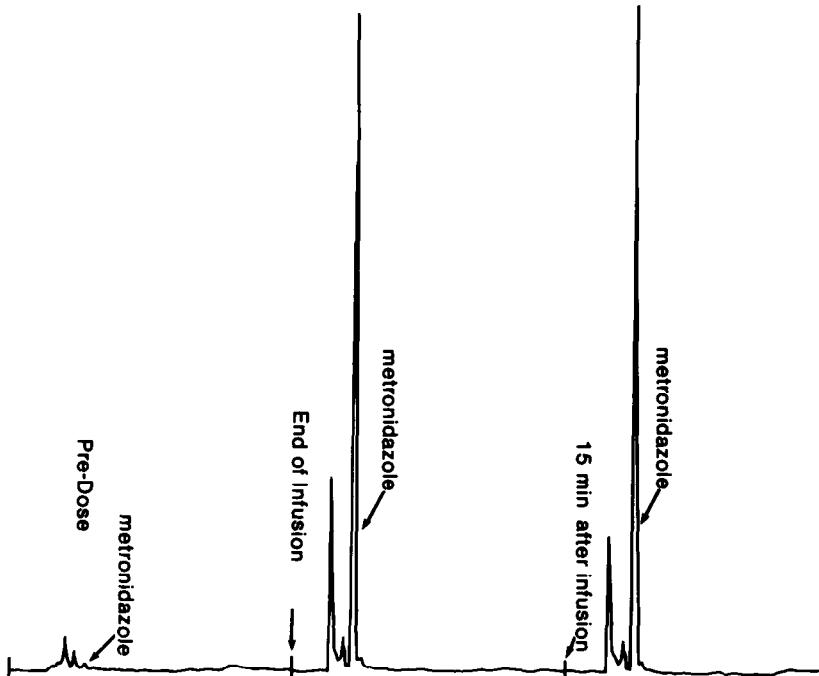


Fig. 1. Typical chromatogram of pre-dose serum, end of infusion (metronidazole serum concentration $4.2 \mu\text{g}/\text{ml}$), 15 min after infusion ($4.3 \mu\text{g}/\text{ml}$) as recorded on a two-pen strip chart recorder. Metronidazole elutes in ca. 2 min.

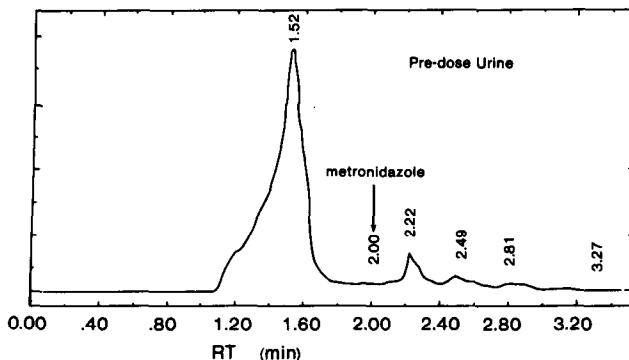


Fig. 2. Typical chromatogram of pre-dose urine sample.

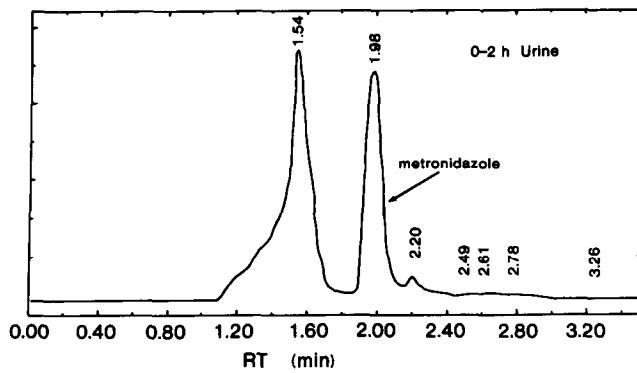


Fig. 3. Typical chromatogram of 0-2-h urine sample, 90 µg/ml.

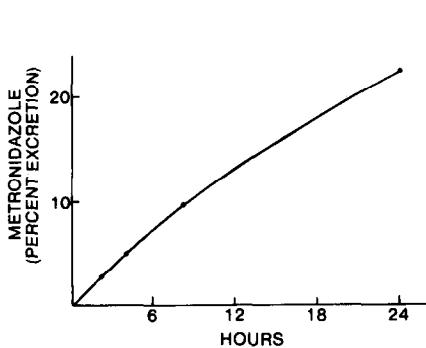


Fig. 4. Cumulative urinary excretion of metronidazole.

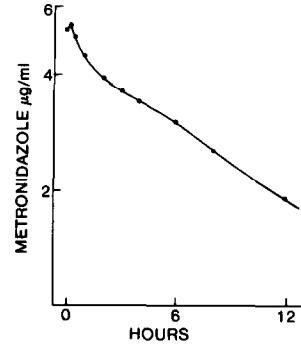


Fig. 5. Serum concentrations of metronidazole.

dose (see Fig. 4). The half-life of metronidazole in serum was 10.9 h (see Fig. 5) which agrees with previously reported data [2].

Reproducibility of six injections of 10 µg/ml metronidazole gave a relative standard deviation (R.S.D.) of 2.8% ($x = 1.03 \cdot 10^5$ area units) and for 100 µg/ml, the R.S.D. was 1.1% ($x = 1.02 \cdot 10^6$ area units). For a series of concentrations from 1 to 100 µg/ml, the correlation coefficient was >0.999 . A plot

TABLE I
RECOVERY OF METRONIDAZOLE IN SERUM AND URINE

Serum ($\mu\text{g/ml}$)	Percent recovered	Urine ($\mu\text{g/ml}$)	Percent recovered
0.1	90.5	1	103
	95.0		100
1.0	95.0	10	94
	97.5		98
5.0	98.0	50	97
	99.0		96
10	101.0	100	99.5
	97.5		96.0
$X = 96.7$		$X = \underline{97.9}$	

passed through the origin. (The R.S.D. of the response normalized for concentration was 2.6%).

Recovery studies in serum and urine are summarized in Table I.

The limit of quantitation for metronidazole in urine was determined to be 0.1 $\mu\text{g/ml}$, and in serum, 0.05 $\mu\text{g/ml}$.

In summary, a HPLC assay for metronidazole in body fluids using a simple sample treatment procedure has been described.

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