DETERMINATION OF METRONIDAZOLE AND ITS TWO MAJOR METABOLITES IN BIOLOGICAL FLUIDS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole; Flagyl®] is an antibacterial agent which has been widely used in the treatment of a variety of anaerobic infections (Templeton, 1977). The major urinary metabolites which have been reported for man are 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole and 2-methyl-5-nitroimidazole-1-acetic acid (Stambaugh, Feo & Manthei, 1968). The analytical methods which are most commonly used to determine this drug in biological fluids are polarography (Kane, 1961), microbiological assay (Levison, 1974) or gas chromatography (Midha, McGilveray & Cooper, 1973; Wood, 1975). The first two methods are non-specific, and the gas chromatographic methods, although specific, are designed for the assay of metronidazole alone, and have a sensitivity which is more appropriate for the measurement of therapeutic levels than for pharmacokinetic studies of metronidazole.

The specific and sensitive high pressure liquid chromatographic (HPLC) method described herein was devised for the simultaneous determination of metronidazole and its two major human oxidative metabolites in pharmacokinetic studies. During the final stages in the preparation of this letter, it was noted that an HPLC method for the quantification of metronidazole and its metabolites in plasma was published by Wheeler, DeMeo, Halula, George & Heseltine (1978). This method is similar in some regards to that described here. However, our method has a considerably lower limit of sensitivity for compounds in plasma, may be used to quantify metronidazole and its metabolites in urine, employs an internal standard for greater accuracy, and has a different sample work-up procedure which is likely to lead to a longer HPLC column life. The extraction and chromatographic procedures used in the assay method are described below.

An aliquot (100 µl) of the HPLC mobile phase (a mixture of 0.1 M diammonium hydrogen phosphate and methanol, 5:1 v/v) containing a known concentration (0.1 to 40 µg/ml) of internal standard [1-(2-hydroxyethyl)-2-ethyl-5-nitroimidazole] was added to heparinized human plasma (5 ml). Ammonium sulphate (3.3 g; prewashed with methyl ethyl ketone) was added and the mixture shaken for 5 min. The pH of the mixture was adjusted to 3.5 with 2 M hydrochloric acid. Methyl ethyl ketone (25 ml) was added and the mixture shaken for a further 5 min, centrifuged and the organic layer removed and retained. This was dried over anhydrous sodium sulphate (5 g; prewashed with methyl ethyl ketone), filtered and evaporated to dryness under vacuum. The residue was dissolved in anhydrous methanol (3 ml), transferred to a vial and evaporated to dryness under a stream of oxygen-free-nitrogen. The residue was dissolved in mobile phase (100 µl) and an aliquot (10 to 25 µl) injected onto the HPLC column.

The extraction method described above was also used for the quantification of the three compounds of interest in urine, except that the quantity of internal standard was increased to a known concentration in

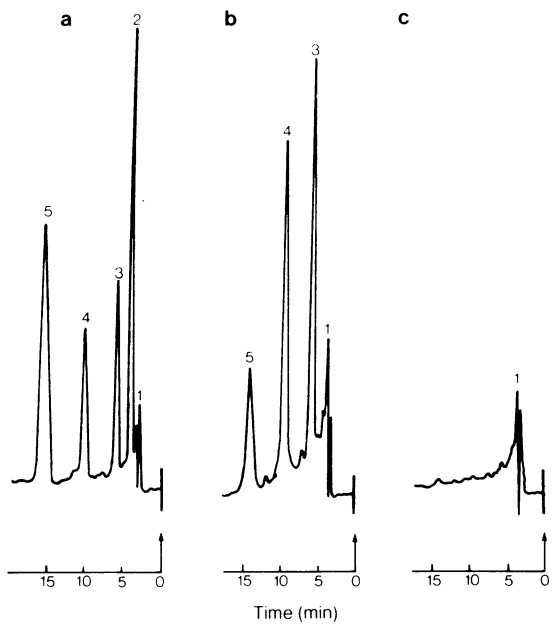

Figure 1 HPLC chromatograms of extracts of:

(a) Human plasma spiked with 500 ng/ml each of metronidazole, 2-methyl-5-nitroimidazole-1-acetic acid, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole, and 1 µg/ml of internal standard, 25 µl injected at a sensitivity of 0.2 Absorbance units f.s.d.

(b) Human plasma collected 48 h after a single oral dose of 500 mg of metronidazole. 15 µl injected at sensitivity 0.05 Absorbance units f.s.d.

(c) Human plasma collected from the same subject as (b) prior to dosing, 25 µl injected at sensitivity 0.05 Absorbance units f.s.d.

Identification of peaks: 1 = solvent front, 2 = 2-methyl-5-nitroimidazole-1-acetic acid, 3 = 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole, 4 = metronidazole, 5 = internal standard.

Table 1 Reproducibility of the assay procedure for metronidazole and its metabolites extracted from plasma or urine

	Coefficient of variation (Mean ± s.d.)		
Compound	*Determined concentration of compound in plasma containing 100 ng/ml of each (ng/ml)*	*Determined concentration of compound in plasma containing 5 μg/ml of each (μg/ml)*	*Determined concentration of compound in urine containing 2.5 μg/ml of each (μg/ml)*
Metronidazole	93 ± 7.3 (7.8%)	5.3 ± 0.11 (2.0%)	2.7 ± 0.06 (2.4%)
2-methyl-5-nitro-imidazole-1-acetic acid	109 ± 11 (10.1%)	5.4 ± 0.32 (5.9%)	2.4 ± 0.21 (8.7%)
1-(2-hydroxyethyl)-2-methyl-5-nitro-imidazole	100 ± 8.3 (8.3%)	5.4 ± 0.17 (3.2%)	2.5 ± 0.09 (3.6%)
Number of replicate determinations	10	10	10

the range 1 to 250 μg/ml and the amount of urine used per assay was 2 ml. Quantities of methyl ethyl ketone, ammonium sulphate and anhydrous sodium sulphate were reduced proportionally.

The HPLC column (stainless steel, 30 cm × 0.3 cm i.d.) was packed with Spherisorb S.10 ODS (Phase Separations Ltd.) Chromatographic conditions employed were as follows: mobile phase flow rate, 0.5 ml/min; pressure, 40 bar; temperature 25°C; u.v. detector λ max, 328 nm; recorder chart speed, 120 mm/h. Under these conditions, the retention times for the three compounds of interest and the internal standard were: 2-methyl-5-nitroimidazole-1-acetic acid, 3.5 min; 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole 5.8 min; metronidazole, 10 min; internal standard, 15 min, each for a column of estimated efficiency of 3000 theoretical plates.

Figure 1 shows the HPLC traces produced when human plasma was extracted and chromatographed as described. The extract of human plasma containing no nitroimidazoles gave no HPLC peaks which would interfere with the determination of metronidazole, 2-methyl-5-nitroimidazole-1-acetic acid and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole at concentrations equal to or greater than 10 ng/ml.

Following the assay of plasma and urine containing known concentrations of each compound, the parameters of the linear regression lines of best fit, constrained to pass through zero, were calculated for each compound in each body fluid. The regression coefficient of each line was used to produce linear standard graphs for metronidazole and the two metabolites.

The reproducibility of the assay procedure for extracts of plasma containing the three compounds of interest was studied at an approximately clinically effective plasma level (5 μg/ml) and at a considerably lower level (100 ng/ml). The results of these studies are given in Table 1. The lowest limits of accurate quantification for these three compounds is in the range 20 to 50 ng/ml. At lower plasma levels, semi-quantitative determinations can be carried out. The limit of detection is variable, but is commonly of the order of 10 ng/ml for all three compounds.

The reproducibility of the assay procedure for extracts of urine was studied at a level of 2.5 μg/ml for each of the three compounds of interest, and the results of this study are reported in Table 1. The lowest limits of accurate quantification of the three compounds of interest are approximately 1 μg/ml for metronidazole and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole and 2.5 μg/ml for 2-methyl-5-nitroimidazole-1-acetic acid, using urine which has been frozen and thawed. In the case of urine, the limiting factor is not the sensitivity of the detector system, but the background of interfering peaks observed when control urine extracts are injected.

The specific assay procedure described herein for the determination of metronidazole and its two major metabolites has been shown to be reproducible at the concentrations studied. The sensitivity of the assay procedure is sufficient to allow the plasma levels of metronidazole to be measured for between six and eight half-lives after the peak plasma concentration has been reached, following administration of a single oral dose of metronidazole (500 mg). Urinary levels of metronidazole and its two major metabolites can be followed for up to 72 h after a similar dose. This method can therefore be used to specifically determine plasma and urinary levels of metronidazole, 2-methyl-5-nitroimidazole-acetic acid and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole in pharmacokinetic

studies aimed at elucidating the clearance kinetics of metronidazole and its major metabolites.

Such studies have been performed, and will be reported in a subsequent publication.

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A STUDY OF THE TRANSFER OF α-METHYLDOPA TO THE HUMAN FOETUS AND NEWBORN INFANT

Methyldopa (Aldomet) has been used for many years for the treatment of hypertension and the main features of its disposition and metabolism in normal and hypertensive adults have been established (Buhs, Beck, Speth, Smith, Trenner, Cannon & Laragh, 1964; Au, Dring, Grahame-Smith, Isaac & Williams, 1972). This antihypertensive agent has also been used for the control of hypertension in pregnancy (Redman, Beilin, Bonnas & Ounsted, 1976), but there appear to be no reports indicating the extent of placental transfer of methyldopa to the foetus or of the levels in the milk of mothers receiving this drug.

This letter describes the results of preliminary studies conducted around the time of delivery, of the concentration of methyldopa in maternal and foetal blood, amniotic fluid and early milk samples from women who have received treatment with the drug during their pregnancy. These investigations were carried out with the approval of the Northwick Park Hospital Ethical Committee and under its rules.

Samples of blood, amniotic fluid and milk were obtained from pregnant women who had received continuous treatment with methyldopa for at least 4 weeks to the time of delivery; the doses ranged from 0.75–2.0 g per day. Maternal and umbilical cord blood samples (10 ml) were collected into heparinized tubes at delivery. The plasma was separated, and after the addition of sodium metabisulphite (0.5 mg/ml), the samples were kept at −20°C until analysed. Samples of amniotic fluid were treated in the same way as plasma. Milk samples, collected between 30 and 60 h after delivery, from three women, were analysed on the day of collection when possible, or refrigerated at +4°C overnight.

The concentration of free and conjugated methyldopa in these fluids was determined with a fluorimetric method of assay essentially the same as that described by Kwan, Foltz, Breault, Baer & Totaro (1976). No blank samples of these fluids were available, but a correction for background fluorescence was made as described by Saavedra, Reid, Jordan, Rawlins & Dollery (1975). The milk was analysed in the same way as plasma except that at the protein precipitation stage, the solution was shaken with 10 ml of ether before centrifuging, to remove lipids. Fluorimetric measurements were made at 330 nm activating and 380 nm emission wavelengths.