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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE NITROIMIDAZOLE AZANIDAZOLE IN HUMAN PLASMA AND URINE

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SUMMARY

Azanidazole can be measured in plasma and urine by reversed-phase high-performance liquid chromatography employing UV detection. Peak mean plasma concentrations of azanidazole, of 267 ng/ml, occurred at 1.5 h after single oral doses to human subjects, and declined with a half-life of 0.8 h. Less than 0.5% of the dose was excreted in the urine as unchanged drug. Metabolites of azanidazole were also detected by the procedures used.

INTRODUCTION

Azanidazole (Triclose®, Fig. 1) is a new nitroimidazole antitrichomonal drug[1]. Nitroimidazoles have been measured in biological fluids by colourimetry[2], thin-layer chromatography–densitometry[3], polarography[2,4] and gas chromatography[5]. These methods suffer from a potential lack of specificity, sensitivity or require derivatisation procedures. More recently, the versatile technique of high-performance liquid chromatography (HPLC) has been applied to the measurement of misonidazole[6,7], metronidazole[7–9] and tinidazole[9,10]. A rapid and sensitive HPLC method has now been

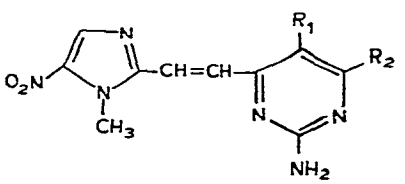


Fig. 1. Structures of azanidazole (R_1 = H, R_2 = H), 5-hydroxyazanidazole (R_1 = OH, R_2 = H), and internal standard (R_1 = H, R_2 = CH_3).

developed for measurement of concentrations of azanidazole (Fig. 1) in plasma and urine and applied to the study of the pharmacokinetics of this drug after single oral doses to human subjects.

EXPERIMENTAL

Azanidazole (4-[(*E*)-2-(1-methyl-5-nitroimidazol-2-yl)vinyl] pyrimidin-2-ylamine, Fig. 1), 5-hydroxyazanidazole (4-[2-(1-methyl-5-nitroimidazol-2-yl)vinyl]-5-hydroxypyrimidin-2-ylamine) and (4-[2-(1-methyl-5-nitroimidazol-2-yl)vinyl]-6-methylpyrimidin-2-ylamine, used as an internal standard) were kindly provided by Istituto Chemioterapico Italiano (Milan, Italy). Standard solutions of azanidazole and internal standard were prepared in methanol at concentrations of 10 μg/ml and 100 μg/ml. Reagents were of analytical grade, and inorganic reagents were prepared in freshly glass-distilled water. Ethyl acetate (Distol reagent, Fisons Scientific Apparatus, Loughborough, Great Britain) was used without further purification. Phosphate buffer was prepared containing potassium dihydrogen orthophosphate (0.5 *M*) adjusted to pH 8 with potassium hydroxide solution.

Plasma extraction procedure

Plasma (1 ml) was spiked with internal standard (200 ng), mixed with orthophosphate buffer (100 μl), pH 8, and the mixture extracted by shaking it with ethyl acetate (5 ml) for 1 min. For samples containing high concentrations of drug, less plasma (0.5 ml) was taken and diluted with "blank" control plasma (0.5 ml). After centrifugation at 2000 *g* for 10 min, the organic layer was carefully transferred to a 10 ml pointed centrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was washed to the bottom of the tube with a small volume of ethyl acetate, which was again evaporated to dryness. The residue was dissolved in methanol (20 μl) and an aliquot (5–20 μl) was injected into the chromatograph.

Urine extraction procedure

Urine (0.5 ml) was spiked with internal standard (4 μg), mixed with orthophosphate buffer (0.5 ml), pH 8, and the mixture extracted by shaking it with ethyl acetate (5 ml) for 1 min. After centrifugation at 2000 *g* for 10 min, the organic layer was carefully transferred to a 10 ml pointed centrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was washed to the bottom of the tube with a small volume of ethyl acetate, which was again evaporated to dryness. The residue was dissolved in methanol (40 μl) and an aliquot (10–40 μl) was injected into the chromatograph.

High-performance liquid chromatography

The chromatograph consisted of a Waters M 6000 A pump (Waters Assoc., Northwich, Great Britain) coupled to a Cecil 212 variable-wavelength spectrophotometer (Cecil Instruments, Cambridge, Great Britain) operated at 368 nm, a $\lambda_{max.}$ for azanidazole. Injection was made by syringe via a Waters U6K, universal injector. The column was constructed of stainless-steel (25 × 0.46 cm I.D.) and prepacked with Partisil 10 ODS/2 (mean particle diameter 10 μm) (Whatman, Maidstone, Great Britain).

Chromatography was performed in reversed-phase mode using a mobile phase of 52% methanol in aqueous potassium dihydrogen orthophosphate (0.1%, w/v). The mobile phase flow-rate was 2 ml/min. Under these conditions internal standard, azanidazole and 5-hydroxyazanidazole had retention times of 11 min, 7 min and 4 min, respectively (Fig. 2).

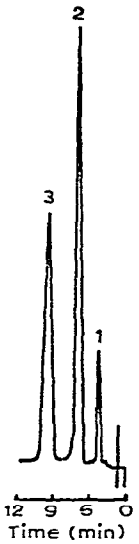


Fig. 2. Chromatogram of reference standards. Column: (25 × 0.46 cm I.D.) prepacked with Partisil ODS/2; flow-rate: 2 ml/min; solvent system, 52% methanol in aqueous potassium dihydrogen orthophosphate (0.1% w/v); detector, UV, 368 nm. Peaks: 1 = 5-hydroxyazanidazole; 2 = azanidazole; 3 = internal standard.

Calibration procedures

Calibration lines were constructed from peak height ratio measurements of azanidazole to internal standard against concentration (Figs. 3 and 4) over the concentration ranges of up to 300 ng/ml in plasma and up to 5 μg/ml in urine. Samples of blank plasma (1 ml) were spiked with azanidazole at concentrations of 20, 50, 100, 200 and 300 ng/ml and with internal standard at 200 ng/ml. Samples of pre-dose urine (0.5 ml) were spiked with azanidazole at concentrations of 1, 2, 3, 4 and 5 μg/ml and with internal standard at 8 μg/ml. Samples were taken through the extraction procedures described previously.

Studies in humans

Plasma and urine samples obtained from 6 human volunteer subjects administered single oral doses of azanidazole (400 mg in a capsule), were analysed by the foregoing procedures. The studies in volunteer subjects were carried out under conditions similar to those described by Brodie *et al.*[11].

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration was repeated on nine occasions from plasma and on five occasions from urine. The precision of the method

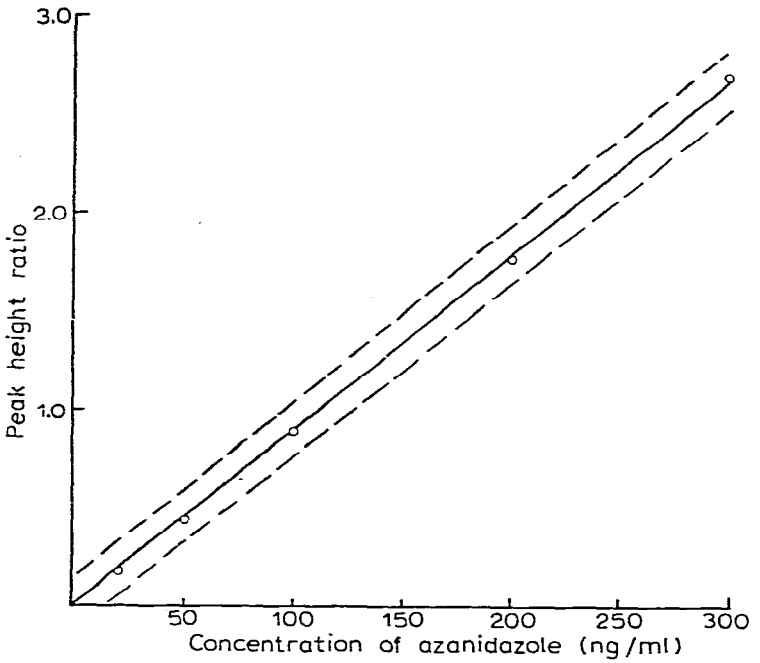


Fig. 3. Calibration line of azanidazole extracted from plasma showing 95% confidence limits.

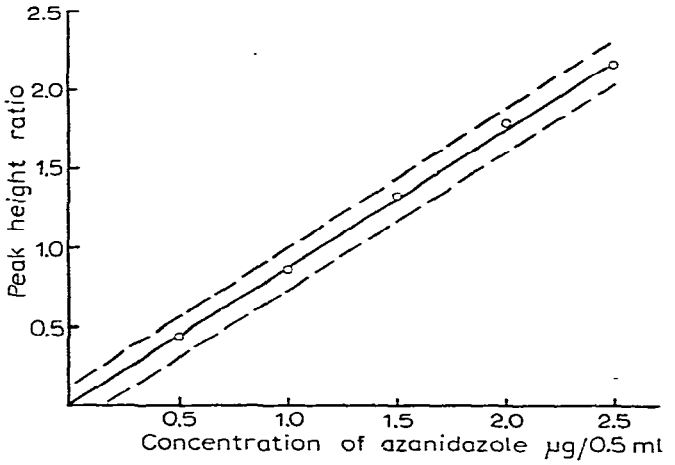


Fig. 4. Calibration line of azanidazole extracted from urine showing 95% confidence limits.

for the measurement of azanidazole was indicated by the coefficient of variation of peak height ratios (Table I) which were ±13%, ±5% and ±4% at 20 ng/ml, 100 ng/ml and 300 ng/ml, respectively, when extracted from plasma (1 ml) and ±3%, ±2% and ±6% at 1 μg/ml, 3 μg/ml and 5 μg/ml, respectively, when extracted from urine (0.5 ml). The coefficient of variation of peak height ratio measurements of a non-extracted mixture of azanidazole and internal standard was ±4% throughout the analysis of all plasma and urine samples.

TABLE I

PRECISION OF THE METHOD FOR THE MEASUREMENT OF AZANIDAZOLE IN PLASMA AND URINE

Concentration added to plasma (ng/ml)	*Coefficient of variation (%)*	*Concentration added to urine (μg/ml)*	*Coefficient of variation (%)*
20	13	1	3
50	6	2	4
100	5	3	2
200	6	4	2
300	4	5	6

Accuracy

The calibration line for the measurement of azanidazole in plasma was constructed from nine replicates at five concentrations over the given range (20–300 ng/ml) and plots of peak height ratios against concentration were linear ($y = a + bx$, where $a = -0.0123$, $b = 0.0090$, correlation coefficient $r = 0.997$) and the value of the intercept was not significantly different from zero ($P > 0.05$). The equation for the line forced through the origin was $y = 0.0089$ (± 0.0001 S.D.) x, where y is the peak height ratio and x is the concentration of azanidazole (ng/ml). The accuracy of the method as defined by the 95% confidence limits of the least squares regression line forced through the origin, *i.e.*, taking the calibration line as an estimate of the concentration of azanidazole in plasma, was $\pm 80\%$, $\pm 12\%$ and $\pm 6\%$ at 20 ng/ml, 130 ng/ml and 300 ng/ml, respectively.

The calibration line for the measurement of azanidazole in urine was constructed from five replicates at five concentrations over the given range (1–5 μg/ml), and plots of peak height ratios against concentration were linear ($y = a + bx$, where $a = 0.0094$, $b = 0.8692$, correlation coefficient $r = 0.995$) and y is the peak height ratio and x is the amount of azanidazole in 0.5 ml of urine. The value of the intercept was shown to be not significantly different from zero. The equation for the line forced through the origin was $y = 0.8743$ (± 0.0080 S.D.) x, where y is the peak height ratio and x is the amount of azanidazole (μg) in urine (0.5 ml). The 95% confidence limits for the least squares regression line forced through the origin were $\pm 31\%$, $\pm 10\%$ and $\pm 7\%$ at 1 μg/ml, 3 μg/ml and 5 μg/ml, respectively.

Recovery

The recovery of internal standard (200 ng/ml), from plasma (1 ml) was determined by comparison of peak height ratio measurements of internal standard to azanidazole of standards taken through the extraction procedure to those injected into the chromatograph without extraction. The mean recovery of internal standard was $94.5\% \pm 1.0$ S.D. ($n = 4$).

The mean recovery of internal standard from urine (0.5 ml) at a concentration of 8 μg/ml was determined similarly and was $90.4\% \pm 8.6$ S.D. ($n = 5$).

The mean recovery of azanidazole from plasma was determined by comparison of peak height ratio measurements of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was $96.6\% \pm 2.3$ S.D. (Table II).

The mean recovery of azanidazole from urine was determined similarly and was 90.8 ± 1.6 S.D. (Table II).

TABLE II

RECOVERY OF AZANIDAZOLE FROM PLASMA AND URINE

Means of nine determinations (plasma) and five determinations (urine) at each concentration.

Concentration added to plasma (ng/ml)	*Recovery (%)*	*Concentration added to urine (μg/ml)*	*Recovery (%)*
20	100	1	91
50	98	2	91
100	95	3	92
200	95	4	92
300	95	5	88
Mean ± S.D.	96.6 ± 2.3	Mean ± S.D.	90.8 ± 1.6

Stability of azanidazole in samples

The stability of azanidazole in plasma and urine under the storage conditions used (−20°) was tested by the re-analysis of 10% of the samples up to one week after the first analysis. No decomposition was detected in either plasma or urine.

Limits of detection

No interfering peaks were present in predose (blank) plasma with retention times similar to azanidazole (Fig. 5). The limit of detection of azanidazole based on instrumental noise was approximately 10 ng/ml.

Concentrations of azanidazole in plasma

After single oral doses of 400 mg azanidazole, a peak of mean concentrations of parent drug of 267 ng/ml occurred at 1.5 h after dosing (Table III). Thereafter mean concentrations declined by an apparent first order process with a half-life of 0.8 h (Fig. 6). The mean concentrations of azanidazole in plasma were below the limit of detection at 6 h after dosing.

A peak was present on chromatograms of extracts of post-dose plasma (Fig. 5) which could not be resolved from 5-hydroxyazanidazole, and the identity of this peak was assigned to the hydroxylated metabolite. However, as no analytically pure sample of 5-hydroxyazanidazole was available, it was not possible to measure accurately concentrations of this metabolite. Another peak was present on chromatograms of extracts of post-dose plasma (Fig. 5) which was resolved from azanidazole and 5-hydroxyazanidazole, and since this peak was not present in extracts of predose plasma, it was presumed to represent a metabolite of azanidazole. Isolation and mass spectrometry of this metabolite suggested that it was possibly an N-oxide of azanidazole (unpublished work). Concentrations of this metabolite could not be measured because its spectral characteristics were not known. No peaks were detectable in any plasma sample withdrawn 24 h after dosing.

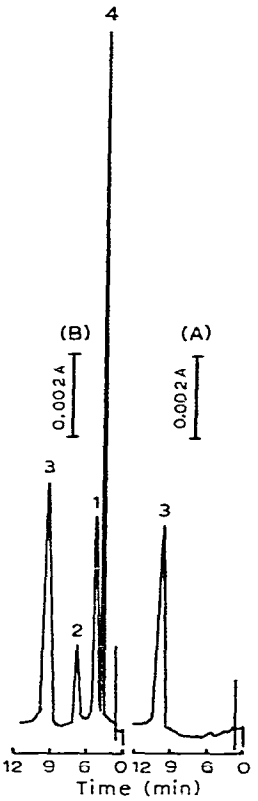


Fig. 5. Chromatograms of (A) predose (blank) plasma, and (B) 3-h post-dose plasma. Conditions as for Fig. 2. Peaks: 1 = 5-hydroxyazanidazole; 2 = azanidazole; 3 = internal standard; 4 = metabolite of unknown structure (N-oxide?).

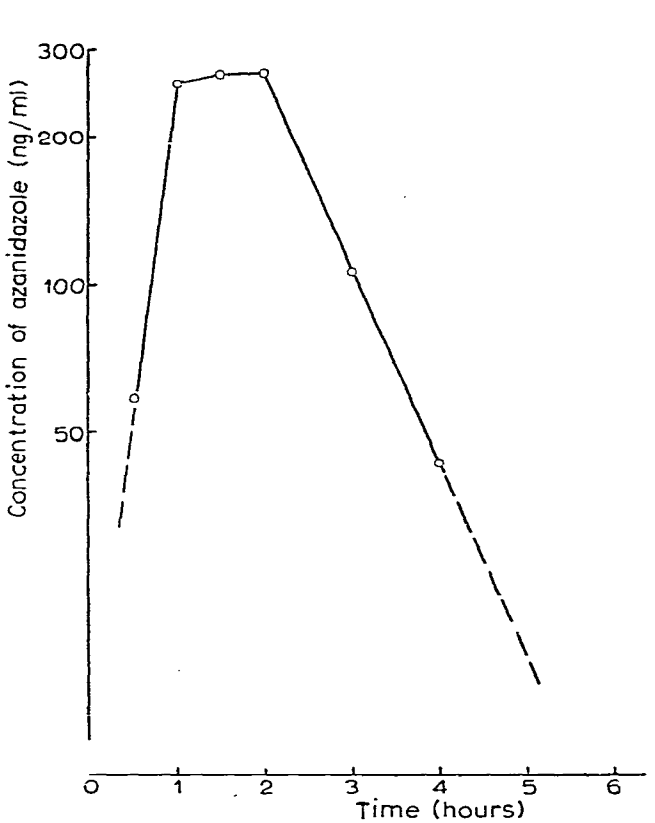
TABLE III

CONCENTRATIONS OF AZANIDAZOLE (ng/ml) IN THE PLASMA OF SIX HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 400 mg

Time (hours)	*Subject No.*						
	1	*2*	*3*	*4*	*5*	*6*	*Mean ± S.D.*
0.5	38	<10	215	103	<10	<10	59 ± 86
1	85	76	396	344	<10	618	253 ± 239
1.5	416	137	180	483	29	355	267 ± 178
2	560	128	85	418	227	175	266 ± 185
3	319	43	34	142	44	46	105 ± 112
4	110	16	16	72	24	17	43 ± 40
6	13	<10	<10	13	<10	<10	<10 —
8	<10	<10	<10	<10	<10	<10	<10 —

Concentrations of azanidazole in urine

During a 9-h period after single oral doses of azanidazole, a mean of 0.18% dose was excreted in the urine (Table IV) as unchanged drug; no unchanged drug was detected in urine collected at times later than 9 h after dosing.



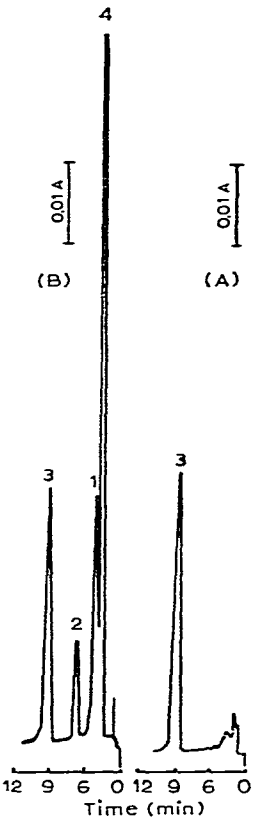


Fig. 6. Mean plasma concentrations of azanidazole during 8 h after an oral dose of 400 mg of drug in a capsule. Semilogarithmic scale.

Fig. 7. Chromatograms of (A) predose urine and (B) 0–3 h urine. Conditions as for Fig. 2. Peaks: 1 = 5-hydroxyazanidazole; 2 = azanidazole; 3 = internal standard; 4 = metabolite of unknown structure (N-oxide?).

TABLE IV

EXCRETION OF UNCHANGED AZANIDAZOLE IN URINE (0–9 h) AFTER SINGLE ORAL DOSES OF 400 mg

Subject No.	*Amount excreted (mg)*	*Dose excreted (%)*
1	0.49	0.12
2	0.67	0.17
3	0.18	0.05
4	0.55	0.14
5	0.87	0.22
6	1.47	0.37
Mean ± S.D.	0.71 ± 0.44	0.18 ± 0.11

Chromatograms of extracts of post-dose urine (Fig. 7) also showed peaks with retention times similar to 5-hydroxyazanidazole and the other postulated plasma metabolite. 5-Hydroxyazanidazole occurred in urine in both free and conjugated forms.

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