High-Performance Liquid Chromatographic Assay for the Antiprotozoal Agent, Tinidazole, in Human Plasma

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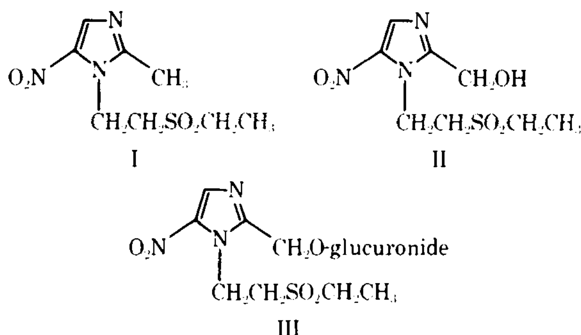
Abstract □ A rapid and specific high-performance liquid chromatographic (HPLC) assay for the quantitative determination of tinidazole in human plasma is described. After a simple extraction procedure, the compound is analyzed by HPLC using a reversed-phase column and a UV detector. Quantitation is accomplished using an external standard, and peak areas are determined with an integrating computer. The average recovery of tinidazole over a concentration range of 0.20–20.0 μg/ml was 86.9 ± 2.8% *SD*. The maximum sensitivity of the assay is approximately 0.10 μg/ml; it is suitable for use in pharmacokinetic studies following administration of therapeutic levels of tinidazole to humans.

Keyphrases □ Tinidazole—high-performance liquid chromatographic assay, human plasma □ High-performance liquid chromatography—analysis, tinidazole in human plasma □ Antiprotozoal agents—high-performance liquid chromatographic analysis in human plasma □ High-performance liquid chromatography—analysis, tinidazole in human plasma

The antitrichomonal agent tinidazole, ethyl-2-(2-methyl-5-nitro-1-imidazolyl ethyl) sulfone (I), is one of the most active compounds in a series of nitroimidazole derivatives (1) active against *Trichomonas vaginalis* (2–5). Attempts to measure tinidazole or other *N*-1-substituted nitroimidazoles in plasma samples have included polarography (6–8), absorptiometry (9, 10), GLC (10), TLC (10, 11), *in vitro* agar diffusion (12), and high-performance liquid chromatography (HPLC) (13).

Quantitation of both tinidazole and metronidazole (7, 8) based on the polarographic assay described by Kane (6) lacks specificity since all drug-related species possessing the nitroimidazole nucleus are similarly detected. The *in vitro* agar diffusion assay (12), the absorptiometry method (9, 10), and the HPLC procedure (13) have maximum sensitivities of 0.5–1.0 μg/ml for the *N*-1-substituted 5-nitroimidazoles. This sensitivity limitation may diminish their usefulness in describing the initial phase of drug absorption or the terminal portion of the elimination phase in humans.

The absorptiometric method and the pulsed polarographic and electron-capture GLC procedures (10) require time-consuming isolation of the intact, parent *N*-substituted nitroimidazole by TLC before quantitation. In a modified TLC–densitometry method (11), direct quantitation of tinidazole and metronidazole was achieved, but



this method is not as rapid as conventional HPLC or GLC, which can be automated to handle a large number of clinical samples.

This report describes a simple and specific HPLC method for the rapid estimation of tinidazole in human plasma at concentrations suitable for pharmacokinetic studies. No interference from two known urinary metabolites (II and III) (14, 15) of tinidazole was encountered.

EXPERIMENTAL

Apparatus—Analyses were performed on a liquid chromatograph[1] operated at ambient temperature and equipped with a differential UV (313 nm) detector[2]. Separations were accomplished on a 30-cm × 3.9-mm i.d. reversed-phase column[3]. Samples were introduced onto the column through a septumless injector[4] with a 25-μl syringe[5]. Chromatograms were traced on a strip-chart recorder[6], and peak area integrations were performed by an integrating computer[7] interfaced with the detector by an analog–digital converter[8].

Reagents and Solvents—Tinidazole (I) and two known metabolites of tinidazole, ethyl[2-(2-hydroxymethyl-5-nitro-1-imidazolyl)-ethyl] sulfone (II) and the *O*-glucuronide conjugate of II (III), were used as received[9]. All chemicals and reagents except methanol[10] were reagent grade. Both 2-butanone and water were distilled before use.

Chromatographic Conditions—The mobile phase was 0.05 *M* pH 7.0 KH_2PO_4–NaOH buffer–methanol (86:14 v/v) with the flow rate adjusted to 2.0 ml/min (~2000 psi). The solvent mixture was prepared daily and degassed under reduced pressure before use.

Extraction Procedure—An aliquot (1.0 ml) of plasma was transferred to a 15-ml vial (16 × 125 mm) fitted with a polytef-lined screw cap and diluted with 2.0 ml of 0.05 *M* pH 7.0 KH_2PO_4–NaOH buffer. This aqueous portion was extracted by vigorous shaking with 7.0 ml of petroleum ether (bp 30–60°). The mixture was centrifuged (2 min at 1500×*g*) to obtain a clear supernate, and the organic (upper) layer was discarded. The aqueous layer was extracted twice with ethyl acetate (7.0 ml) by manual shaking for 1 min, and the phases were separated by centrifugation at 1500×*g* for 3 min.

The organic layers were removed with a Pasteur pipet, pooled, taken to dryness under a nitrogen stream in a water bath (55°), and then transferred to a 2.5-ml conical centrifuge tube using ethyl acetate. The ethyl acetate was concentrated as described, the residue was dissolved in 2-butanone (0.1–0.5 ml), and aliquots (5–20 μl) were injected onto the HPLC column.

Extraction Efficiency—Blood from several untreated volunteers was drawn into heparinized containers[11] and centrifuged to generate a plasma pool from which an extraction efficiency was established. Known amounts of tinidazole dissolved in methanol were added to each of six 11.0-ml drug-free plasma pool samples to obtain concentrations of 0.20, 0.39, 1.93, 4.82, 9.82, and 20.0 μg/ml. The plasma pools were thoroughly mixed on a tube rotator for 30 min. Aliquots (1.0 ml) of plasma from each pool were transferred to 15-ml screw-capped vials and stored frozen (−20°) until extracted as described.

[1] Model ALC 204, Waters Associates, Milford, Mass.
[2] Model 440, Waters Associates, Milford, Mass.
[3] μBondapak/phenyl, Waters Associates, Milford, Mass.
[4] Model U6K, Waters Associates, Milford, Mass.
[5] Pressure-Lok B-110, Precision Sampling Corp., Baton Rouge, La.
[6] Servo/riter II, Texas Instruments, Houston, Tex.
[7] Model 3352B, Hewlett-Packard, Avondale, Pa.
[8] Model 18652A, Hewlett-Packard, Avondale, Pa.
[9] Tinidazole and the metabolite standards were synthesized and supplied by Pfizer Ltd., Sandwich, Kent, England.
[10] Distilled in glass, Burdick & Jackson Laboratories, Muskegon, Mich.
[11] Vacutainers, Becton-Dickenson, Rutherford, N.J.

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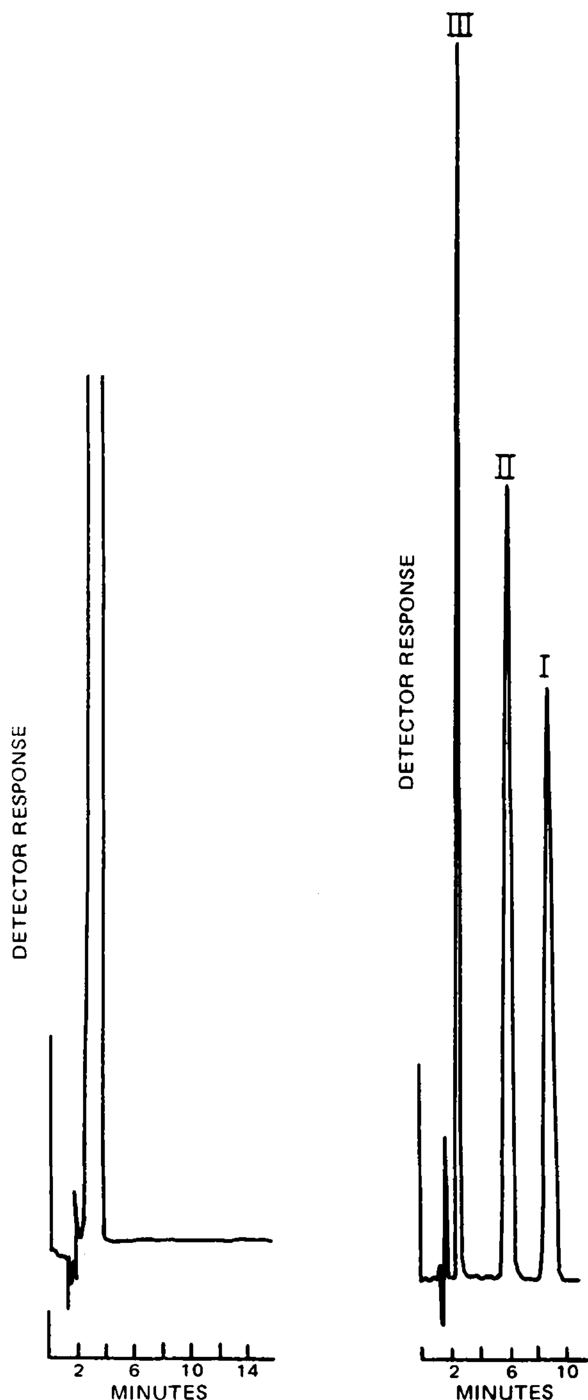

Figure 1—*Liquid chromatogram of an ethyl acetate extract from drug-free plasma. The mobile phase consisted of 0.05* M *KH_2PO_4-NaOH buffer-methanol (86:14 v/v) with the flow rate adjusted to 2.0 ml/min.*

Figure 2—*Liquid chromatogram of a mixture of tinidazole (I), II, and III. Approximately 0.2 μg of each standard was injected for analysis.*

Since the prepared drug concentrations ranged over two orders of magnitude (0.20–20.0 μg/ml), varying volumes of the final 2-butanone dilutions were injected for analysis by HPLC. Typically, when the drug concentration approached 0.2 μg/ml, one-fifth (20 μl/100 μl) of the final dissolved residue volume was analyzed; at a concentration of 20 μg/ml, only one-one hundreth (5 μl/500 μl) of the final dissolved residue was injected. All samples were analyzed in duplicate. After chromatography, quantitation of tinidazole was accomplished by use of the external standard method.

Calibration and Standard Preparation—Tinidazole (10.0 mg) was dissolved in 2-butanone and diluted in a 10.0-ml volumetric flask to 1.0

Table I—Recovery of Tinidazole (n = 11) from Human Plasma at Various Concentrations

Theoretical Plasma Concentration, μg/ml	Average Observed Plasma Concentration, μg/ml	Average Recovery[a], %
0.20	0.18	89.8 ± 3.7
0.39	0.34	88.2 ± 3.3
1.93	1.64	85.2 ± 2.3
4.82	4.16	86.2 ± 2.6
9.82	8.20	83.5 ± 2.5
20.0	17.7	88.6 ± 2.5
		$\bar{x}$ = 86.9 ± 2.8[b]

[a] $\bar{x}$ ± *SD*. [b] *CV* = 3.2%.

μg/μl. Two additional stock solutions were prepared containing 0.10 and 0.01 μg/μl of tinidazole by serial dilution of the 1.0-μg/μl stock solution with 2-butanone. Since the external standard method prepared from the software section of the computer assumes a linear relationship between micrograms injected and peak area (microvolt-seconds as reported by the integrating computer), this relationship was initially evaluated by repeated ($n \geq 5$) injections of 0.25, 0.20, 0.15, 0.10, 0.05, and 0.025 μg of tinidazole from the 2-butanone stock solutions. Thereafter, an average calibration response factor was established by the computer following triplicate injections equivalent to 0.15 μg of tinidazole.

Specificity—Assay specificity was determined by analyzing a methanolic solution containing a mixture (0.2 μg) of tinidazole and two of its known urinary metabolites (II and III). A portion of an extract (20 μg/100 μl) from a drug-free plasma sample was evaluated chromato-

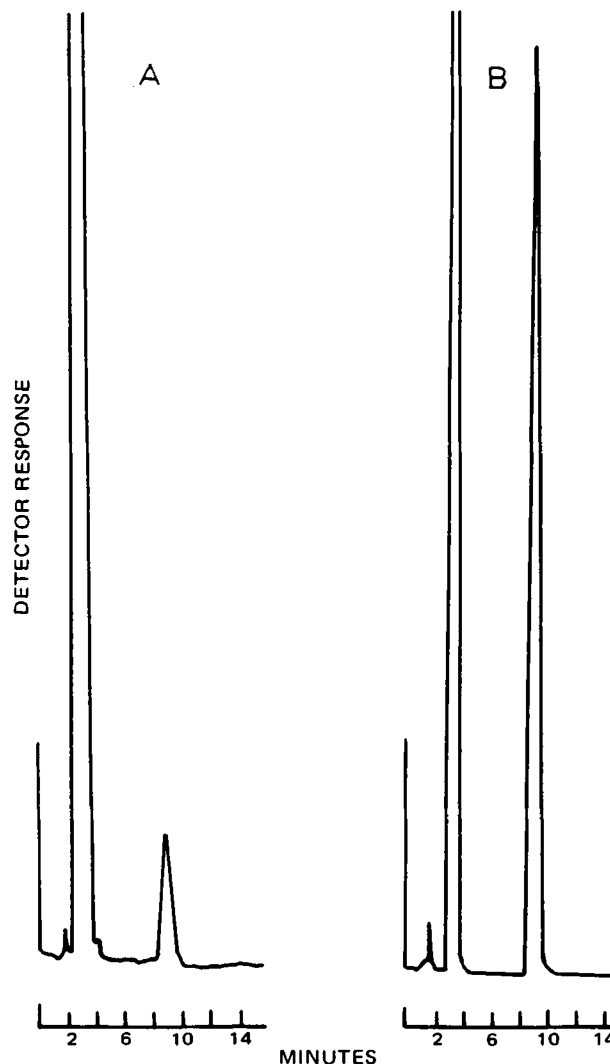

Figure 3—*Representative chromatograms of ethyl acetate extracts from human plasma spiked with 0.2 (A) and 20.0 (B) μg/ml of tinidazole. Detector response was at 0.005 aufs.*

graphically for the presence of any extractable ethyl acetate UV-absorbing (313 nm) material that might interfere with tinidazole measurement.

RESULTS AND DISCUSSION

A successful chromatographic assay for drug levels in the large number of plasma samples generated from a clinical study would provide highly reproducible drug recovery, specificity, sensitivity, precise measurement, and rapid throughput of samples.

The average recovery of drug from plasma samples to which tinidazole had been added was 86.9 ± 2.8% *SD* (Table I). Linear regression analysis of percent recovery *versus* plasma concentration revealed no statistically significant slope (β) ($p = 0.7431$). These data, therefore, suggest that there is no concentration dependence on extraction efficiencies over the range of expected plasma drug levels (10–12).

Tinidazole was monitored at 313 nm since its λ_{max} was observed in methanol at 310 nm ($\epsilon = 9070$). Ethyl acetate extracts from drug-free plasma were free of interfering UV- (313 nm) absorbing peaks (Fig. 1). Preextraction with petroleum ether eliminated nonpolar material that would otherwise be highly retained on this reversed-phase system. Little or no loss of tinidazole was encountered in this step.

Baseline resolution among tinidazole, II, and III was achieved under the chromatographic conditions described (Fig. 2). The observed retention times (t_r) for III, II, and I were 2.73, 6.12, and 8.96 min, respectively. Representative chromatograms of plasma extracts containing 0.20 and 20.0 μg/ml of tinidazole are shown in Fig. 3.

Linear regression analysis of the curve described by plotting microvolt-seconds (area) *versus* micrograms injected indicated a linear fit of the data ($r^2 = 0.9994$) from 0.025 to 0.25 μg. The slope was 474.75 μv-sec/ng; the intercept, which was not significantly different from zero ($p < 0.05$), was 1512 μv-sec. Over 2 months, the slope of this line demonstrated little change, with a coefficient of variation equal to 3.83% ($n = 16$).

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Thermal Characterization of Citric Acid Solid Dispersions with Benzoic Acid and Phenobarbital

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Abstract □ The glass transition temperatures of citric acid glass were determined by differential scanning calorimetry to be 10.2 and 13.5° for *in situ* and bulk-prepared samples, respectively. Mechanical stress on citric acid glass induced foci for crystallization. Benzoic acid addition to citric acid glass decreased its glass transition temperature while phenobarbital addition increased its glass transition temperature, the latter forming a glass solution.

Keyphrases □ Glass transition temperature—citric acid glass, benzoic acid, mechanical stress, phenobarbital □ Citric acid—glass formation, glass transition temperature, benzoic acid, phenobarbital □ Glass formation—citric acid, benzoic acid, phenobarbital □ Phenobarbital—glass formation with citric acid

A glass is generally defined as a noncrystalline solid formed by continuous hardening or solidification of a liquid (1, 2). The use of organic compounds capable of glass formation to reduce the particle size of a drug and to increase its dissolution and absorption rates was first suggested by Chiou and Riegelman (3). They proposed the formation of a glass dispersion of a poorly water-soluble drug and a physiologically inert carrier such as citric acid. The concepts involved in glass solution and glass suspension formation were discussed previously (4).

Glass dispersions are metastable and, as a result, exhibit rapid dissolution. In a study with griseofulvin (3), the citric acid–griseofulvin glass solution had the fastest dissolution rate of the solid dispersion systems examined. However, griseofulvin decomposition occurred in the presence of citric acid during manufacturing. Citric acid was also an unacceptable sulfabenzamide carrier (5). As with griseofulvin, thermal drug degradation occurred during sample preparation.

Summers and Enever (6–8) studied citric acid–primidone glass dispersions and found the glass solutions formed to be unstable and rapidly devitrified. The devitrified dispersion systems, however, still exhibited more rapid dissolution than the pure drug or drug and carrier mixtures. The citric acid glass transition temperature increased with increasing primidone concentration (8). The

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