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Activity of Metronidazole and its Hydroxy and Acid Metabolites Against Clinical Isolates of Anaerobic Bacteria

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Susceptibility of clinical isolates of anaerobic bacteria to metronidazole and its two oxidation products, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (the "alcohol" metabolite) and 2-methyl-5-nitroimidazole-1-acetic acid (the "acid" metabolite), were determined by the agar dilution technique. Results disclosed that the alcohol metabolite, although less active than metronidazole, inhibited the organisms tested at levels considered susceptible for metronidazole. The acid metabolite was less active, not inhibiting the organisms at levels within the susceptible range. In other studies, mixtures of known concentrations of metronidazole and the metabolites were assayed in a bioassay system used to measure metronidazole levels. These studies showed that the bioassay will measure metronidazole or the alcohol metabolite; the acid metabolite is not measured at levels achieved in clinical specimens. Since the activity of the alcohol metabolite is comparable to that of metronidazole, we feel that microbiological assays can be used for therapeutic monitoring of metronidazole levels in clinical situations.

Metronidazole is a nitroimidazole compound with bactericidal activity against a broad range of anaerobic bacteria. Its broad spectrum against anaerobic bacteria and its rapid bactericidal action make it a particularly attractive agent for use in infections in which these microorganisms are dominant pathogens or in which anaerobic bacteremia is present.

Additional reasons which favor the increased use of metronidazole are its excellent penetration into closed-space infections, particularly anaerobic central nervous system infections (5), and the recent reports of resistance among anaerobic bacteria to clindamycin, a widely used antibiotic with a similar spectrum of activity (1).

The pharmacokinetics of metronidazole in humans are not completely established, but it is known that the drug is metabolized, presumably in the liver, to two oxidation products: 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (the "alcohol" or hydroxy metabolite) and 2-methyl-nitroimidazole-1-acetic acid (the "acid" metabolite) (3). Plasma levels of metronidazole are occasionally increased in severe renal disease (2) and may also be prolonged in patients with severe liver disease resulting in accumulation of the drug. (Searle Laboratories, unpublished data).

Because of incompletely defined pharmacokinetics and the potential necessity to measure levels in patients with renal or liver impairment, both biological and chromatographic assays have been developed. Using a high-pressure liquid chromatographic (HPLC) assay, we have noted that the sera of patients receiving metronidazole contain two or three peaks corresponding to metronidazole and the alcohol and acid metabolites. The alcohol is often present in substantial quantities (range, 2 to 23 μg/ml), whereas the acid is usually seen in very small amounts. The biological activity of these metabolites is not well characterized, although the antibacterial activity of the alcohol is said to be less than that of the parent compound (9). If these metabolites are indeed less active against clinical isolates but are active against the very sensitive strains used for biological assay of metronidazole levels in serum, then serum levels by bioassay may represent falsely elevated estimations of metronidazole when, in fact, they represent one or both metabolites.

We are reporting the susceptibility of unselected clinical isolates of anaerobic bacteria to metronidazole and its alcohol and acid metabolites. In addition, we have looked at combinations of metronidazole and the metabolites in the biological assay system which we use to measure metronidazole levels.

MATERIALS AND METHODS

Bacteria. Anaerobic bacteria were isolated from unselected clinical specimens in the Clinical Microbiology Laboratory of the Loyola University Foster G. McGaw Hospital. Specimens were incubated in an

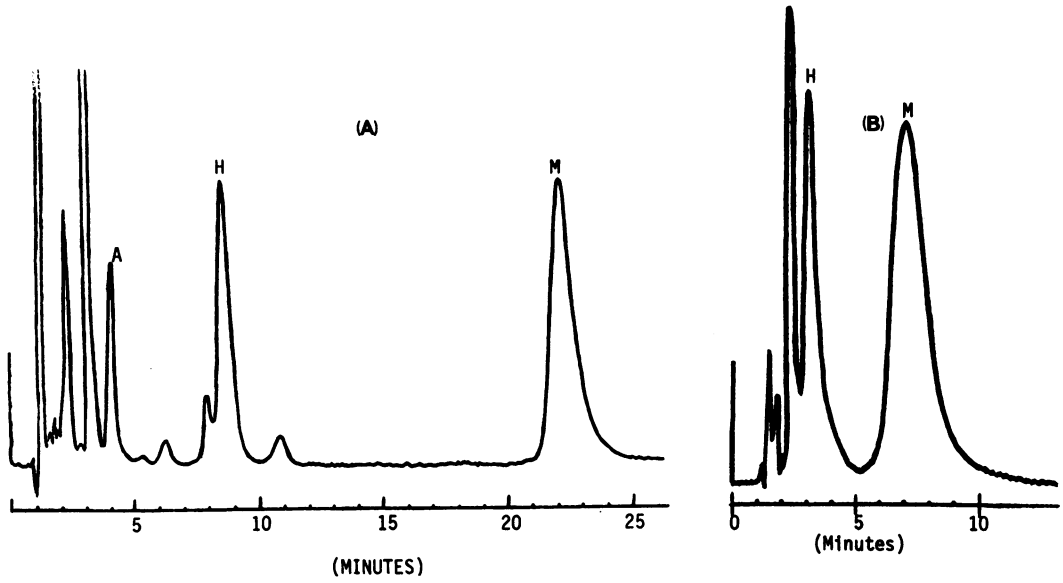

FIG. 1. Chromatographic tracings of metronidazole and metabolites in serum. (A) Tracing showing metronidazole (M), the alcohol (hydroxy) metabolite (H), and the acid metabolite (A); (B) tracing of the condensed assay in which the acid peak is not seen.

anaerobic chamber (Forma Scientific), and isolates were identified by conventional techniques as outlined in the VPI Anaerobe Laboratory Manual (4).

Susceptibility testing. Minimum inhibitory concentrations (MICs) of metronidazole and its two metabolites (kindly supplied by L. Hunt, Searle Research Division, Chicago, Ill.) were determined by the agar dilution technique on Wilkins Chalgren Agar and by using a Steers Replicator. The method is that outlined by The National Committee for Clinical Laboratory Standards (7). The plates were incubated in the anaerobic chamber.

Metronidazole levels. Biological assay of metronidazole and its metabolites was performed by using *Clostridium perfringens* (kindly supplied by Nilda Jacobus, Tufts University Anaerobe Laboratory, Boston, Mass.) in 5% sheep blood. For these studies, known concentrations of metronidazole and its metabolites, either alone or in combination, were added to normal human serum. The following concentrations were used: metronidazole, alcohol metabolite, and acid metabolite, each at 64, 32, 8, and 4 μg/ml; metronidazole plus alcohol metabolite at 32:32 μg/ml, 32:4 μg/ml, and 4:32 μg/ml; and metronidazole plus acid metabolite at 32:32 μg/ml and 32:4 μg/ml. Duplicate samples of each concentration were tested. Zones of inhibition of hemolysis were measured after 4 to 6 h of incubation in the anaerobic chamber (6).

Metronidazole levels were measured by HPLC, using a modification of the method of Nilsson-Ehle et al. (8). Briefly, 0.5 ml of serum was mixed with 0.5 ml of 5% perchloric acid and carefully mixed on a Vortex mixer for 10 s. After standing for 10 min at room temperature, the mixture was centrifuged for 15 min at 1,000 × *g*, and the supernatant was filtered through a 0.45-μm cellulose triacetate filter (Gelman GA-6; Gelman Sciences, Inc.). A 20-μl volume of the filtrate was injected onto the column. The mobile phase was 100% acetate buffer at pH 4.8 and was pumped at a rate of 2.5 ml/min.

Chromatography was accomplished by using an Altex model 110-A pump. The column was a Whatman Partisil 10 ODS-3 C_{18} type reverse phase (25 by 0.46 cm) with a guard column packed with CO:PELL ODS (C_{18} groups bonded to 30- to 38 μm glass beads; Whatman, Inc.). A Rheodyne 20-μl loop injector was used. Metronidazole and its metabolites were detected on an LDC-Spectromonitor III UV variable wavelength detector at 313 nm, and peaks were recorded on a paper chart recorder. Peak heights for metronidazole and its metabolites were measured. Serial twofold dilutions of metronidazole and each of the metabolites

TABLE 1. Representative serum levels of metronidazole and its alcohol metabolite as measured by HPLC

Patient no.	Serum level (μg/ml)	
	Metronidazole	Alcohol
1	7.6	10.2
2	16.8	11.0
3	8.52	4.57
4	14.9	21.4
5	28.3	22.9
6	23.8	15.5
7	10.5	12.7
8	19.85	5.2
9	15.42	3.56
10	18.00	6.4
11	11.1	5.9
12	22.8	7.4

were freshly prepared on the day of assay in normal human serum in concentrations of 64 to 1 μg/ml. These standards were processed for chromatography in a manner identical to the patient specimens.

RESULTS

Serum levels of metronidazole. Figure 1A shows a chromatogram of a serum specimen in which the acid, alcohol, and metronidazole peaks are clearly shown. Levels of the acid metabolite were generally less than 0.5 μg/ml, although they were occasionally above that level (Fig. 1A), and to shorten the time of the assay, the procedure was modified in such a way that the acid metabolite peak was lost in the solvent front (Fig. 1B). Serum levels of metronidazole and the alcohol metabolite from a group of 12 patients are shown in Table 1. The levels of the metabolite were generally high and in some cases exceeded the level of metronidazole in the same specimen. The ratio of the metabolite to metronidazole could not be related to time after dose in this small group of patients.

Susceptibility of clinical isolates to metronidazole and its metabolites. The results of agar dilution susceptibility tests are shown in Table 2. All of the isolates tested were sensitive to low levels of metronidazole. For each group of organisms, the MIC for 90% of the isolates (MIC_{90}) for metronidazole and the alcohol metabolite were within one dilution of each other, with the exception of the *Clostridium* species other than *C. perfringens*, in which there were two dilutions separating the MIC_{90} for the two compounds. Note also that the MICs for the alcohol metabolite were well within the range of susceptibility of metronidazole. On the other hand, the acid metabolite showed considerably less activity against these isolates. The MIC_{90} was five- to eightfold higher for the acid metabolite than for metronidazole. These higher MICs would be considered resistant.

Biological assay of metronidazole. Various concentrations of metronidazole and of both metabolites, alone and in combination, were tested with the anaerobic assay, using *C. perfringens*. Figure 2 shows a representative assay plate. Zones of inhibition of hemolysis are seen around the wells containing metronidazole and each of the metabolites. The zone diameter around the alcohol metabolite is nearly as large as the zone around a similar concentration of metronidazole

TABLE 2. Susceptibility of clinical isolates of anaerobic bacteria to metronidazole and to the alcohol and acid metabolites[a]

Organism (no. of isolates)	Range (μg/ml)	MIC_{50}	MIC_{90}
Bacteroides fragilis (27)			
Metronidazole	<0.125–0.5	0.25	0.5
Hydroxy metabolite	0.25–1	0.5	1
Acid metabolite	4–16	16	16
Bacteroides fragilis group[b] (27)			
Metronidazole	<0.125–0.5	0.25	0.5
Hydroxy metabolite	0.25–1	0.5	1
Acid metabolite	4–16	8	16
Clostridium perfringens (17)			
Metronidazole	<0.125–0.5	0.25	0.5
Hydroxy metabolite	0.25–1	0.5	1
Acid metabolite	8–32	16	32
Other *Clostridium* species[c] (10)			
Metronidazole	0.125–1	0.125	0.5
Hydroxy metabolite	0.25–2	0.5	2
Acid metabolite	2–64	4	16
Gram-positive cocci[d] (24)			
Metronidazole	<0.06–0.5	0.125	0.25
Hydroxy metabolite	<0.06–0.5	0.125	0.25
Acid metabolite	0.25–16	4	16

[a] MIC_{50}, MIC for 50% of isolates tested; MIC_{90}, MIC for 90% of isolates tested.
[b] Includes 4 *B. distasonis* isolates, 2 *B. ovatus* isolates, 8 *B. thetaiotaomicron* isolates, and 13 *B. vulgatus* isolates.
[c] Includes four isolates of *C. difficile*.
[d] Includes 8 *Peptococcus asaccharolyticus* isolates, 10 *Peptococcus prevotii* isolates, 1 *Peptostreptococcus anaerobius* isolate, and 1 *Peptostreptococcus micros* isolate.

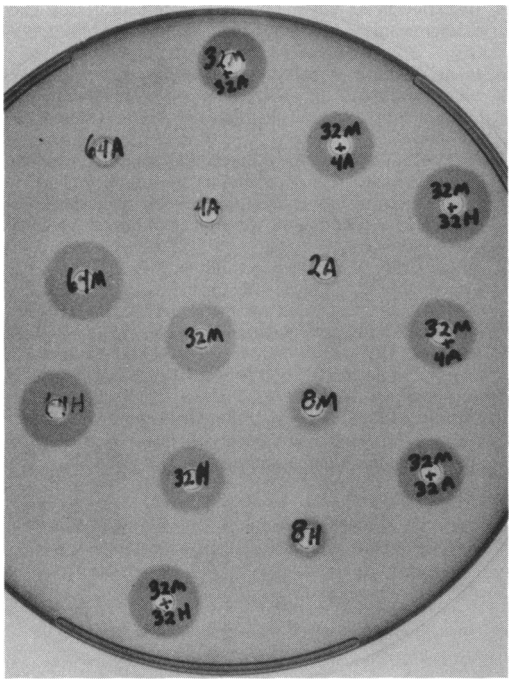

FIG. 2. Microbiological assay of levels of metronidazole (M), the acid metabolite (A), and the alcohol (hydroxy) metabolite (H). Numbers indicate concentration (in micrograms per milliliter) of the substance tested, either alone or in dual combinations.

(11.8 versus 13.2 mm for 32 μg/ml). On the other hand, very high concentrations of the acid metabolite resulted in almost imperceptible zones. Combining metronidazole and one of the metabolites resulted in a zone diameter equivalent to the larger zone produced by similar concentrations of the two components of the mixture when tested alone. For example, the zone diameter for the mixture containing 32 μg of the metronidazole and 4 μg of the alcohol metabolite per ml was equal to the zone with 32 μg of metronidazole per ml alone, whereas the zone diameter for the mixture containing 4 μg of metronidazole and 32 μg of the alcohol metabolite per ml was equal to the zone with 32 μg of the alcohol metabolite per ml alone. Therefore, serum levels of metronidazole determined by bioassay methods will reflect metronidazole or the alcohol metabolite, depending on which species predominates in the sample.

DISCUSSION

It is apparent from the unselected samples of serum submitted to our laboratory for metronidazole levels that significant concentrations of both metronidazole and the alcohol metabolite are detected when HPLC methods are used. The significance of the presence of the alcohol metabolite and the acid metabolite (although generally present in very low concentrations) on the biological activity of metronidazole was not appreciated, although some authors had stated that the metabolites were less active (9).

The results of our studies on the susceptibility of clinical isolates of anaerobic bacteria to metronidazole and the metabolites do not support this previous conclusion. In fact, when tested against our unselected clinical isolates, the activity of the alcohol metabolite was within one dilution of that of the parent compound and well within the range of susceptibility (≤1 μg/ml) for metronidazole. On the other hand, the acid metabolite was less active, and the MICs were outside the susceptible range for metronidazole. Certain pathogenic groups of anaerobic bacteria, namely *Bacteroides melaninogenicus*, *Peptostreptococcus* spp., and *Fusobacterium* spp., are not represented in our results. This is related to the difficulty encountered in cultivating these microorganisms on the media recommended by the National Committee for Clinical Laboratory Standards for agar dilution susceptibility testing of anaerobic bacteria. Susceptibility testing of these groups with supplemented Wilkins Chalgren Agar is planned.

The serum levels measured by HPLC indicated that in certain patients, high levels of the alcohol metabolite were present. Of the two oxidized metabolic products measured in serum samples, the alcohol metabolite was present in much greater quantity and thus was the predominant metabolite. The results of susceptibility tests further disclosed that this dominant circulating metabolite was an active compound and should provide antimicrobial activity comparable to metronidazole itself. This situation differs from that of cephalothin, in which certain cephalothin-sensitive organisms are resistant to its major circulating metabolite (10).

Many laboratories are not able to perform metronidazole levels by HPLC and use biological assays to measure the drug. Our results with the anaerobic *C. perfringens* assay show that the assay will measure either metronidazole or the alcohol metabolite but will not indicate which of these two species is measured. Since the metabolite which is measured in the biological assay has antimicrobial activity equivalent to that of metronidazole, an assay which can discriminate between metronidazole and the alcohol metabolite may not be necessary for therapeutic drug monitoring. In laboratories in which HPLC is not available, we recommend continuing use of the microbiological methods.

Finally, we are not aware of studies on the pharmacokinetics of the metabolites of metronidazole in normal or diseased patients in whom

metabolism may be impaired, nor are there studies on tissue levels of the metabolites. We are attempting to answer these very important questions at the present time.

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