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N-oxidation: a possible route of tinidazole metabolism in man

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Summary

Treatment of tinidazole with a mixture of hydrogen peroxide and acetic acid, or liver homogenate preparations, yields the *N*-3 oxide. This was identified by thin-layer chromatographic analysis on silica gel G, R_f 0.6, using ethanol–chloroform–ammonia (50:49:1) as solvent, and by chemical reduction with sulphur dioxide. UV spectrophotometry and high performance liquid chromatography (HPLC) gave an R_T of 0.55 min using Pye Unicam apparatus equipped with a UV detector at 330 nm, a reversed-phase RP 18 (10 μm) column which was 12.5 cm long, a mobile phase of methanol–0.005 M KH_2PO_4 (pH 4) (20:80, v/v) and a flow rate of 2 ml/min. In-vitro metabolic *N*-oxidation was achieved by incubating the parent drug, tinidazole, with rat liver homogenates fortified with cofactors at 37°C. HPLC analysis of blood and urine samples from healthy volunteer subjects who took a single oral dose of tinidazole showed the presence of an in-vivo *N*-oxidation metabolite of the drug. The identical physico-chemical characteristics of the synthetic and biologically produced tinidazole *N*-oxide strongly suggest that tinidazole, a tertiary amine drug, undergoes metabolic *N*-oxidation.

Résumé

Le traitement de tinidazole avec un mélange d'eau oxygénée et d'acide acétique ou des préparations homogènes du foie donne l'oxyde-*N*3 identifiable par l'analyse chromatographique de couche mince sur le colloide de silice G R_f 0.6 en éthanol–chloroforme–ammoniaque (50:49:1), par la réduction chimique avec l'anhydride sulfureux, la spectrophotometrie UV et par la chromatographie liquide hautement performant (HPLC) R_T 0.55 min. Pye Unicam équipe du détecteur UV 330 nm. La colonne RP 18 (10 μm) était 12.5 cm de long et le taux d'écoulement de la phase mobile méthanol–0.005 M KH_2PO_4 (pH 4) (20:80, v/v) était 2 ml/min. L'oxydation métabolique *in vitro* a été réalisée par l'incubation du produit tinidazole avec des homogènes du foie de rat fortifiés avec des cofacteurs à 37°C. L'analyse HPLC des échantillons du sang et de l'urine des volontaires en bonne santé qui ont pris une dose simple de tinidazole ont montré la présence de oxydation-*N* métabolite *in vivo* du produit. Les caractéristiques physico-chimiques identiques du synthétique et du tinidazole oxyde-*N* produit biologiquement suggèrent fortement que le tinidazole, une drogue amine tertiaire, subit l'oxydation-*N* métabolique.

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Introduction

Tinidazole (Fig. 1) is a potent antiprotozoal agent used successfully in the treatment of amoebiasis, trichomoniasis and giardiasis [1]. Like many other nitroimidazole derivatives

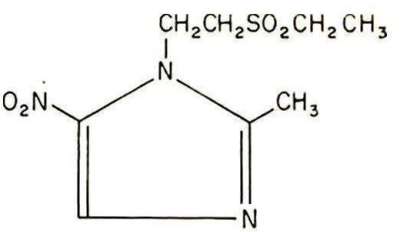

Fig. 1. The structure of tinidazole.

its potential in the management of some anaerobic bacterial infections has been speculated [2,3]. The activity of tinidazole and its hydroxy metabolite against *Gardnerella vaginalis (Haemophilus vaginalis)* was reported in 1982 by Shanker and Munro [4].

The exact mechanism of antiprotozoal action of tinidazole is not clear. Apparently all the drug's biological activities are related to the reduction of its nitro group [5]. The nature of tinidazole metabolites has remained rather unclear until recently [6]. Tinidazole has two tertiary amine centres, and the possibility of *N*-oxidation by microsomal enzymes (*in vitro* and *in vivo*), which hitherto has not been reported, has been investigated.

Materials and methods

Tinidazole was a generous gift from Pfizer Nig. Ltd. (Lagos, Nigeria). Glacial acetic acid, chloroform, ethanol and methanol were all analytical reagent grade and were obtained from BDH (Poole, U.K.). Nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide, glucose-6-phosphate (G-6-P), potassium dihydrogen orthophosphate, sodium hydroxide and magnesium chloride were all also supplied by BDH and were used without further purification. White rats (Sprague-Dawley) were obtained from the animal house of the College of Medicine, University of Lagos. Homogenization was achieved with an Ultra Turrax homogenizer and ultracentrifugation was performed on a Sorvall RC2B model at 10,000 *g*.

Synthesis of tinidazole N-*oxide*

A mixture of tinidazole solution in methanol (0.5 g/30 ml), Glacial acetic acid (10 ml) and hydrogen peroxide (20 ml, 30% H_2O_2) was kept in the dark and stirred magnetically for 24 h. Excess hydrogen peroxide was decomposed by the addition of manganese dioxide (approximately 200 mg) and the mixture shaken for about 10 min. The reaction mixture was filtered (Whatman paper No. 3) and the filtrate concentrated *in vacuo* by means of rotary evaporator. The concentrate was taken up in a dilute solution of aqueous Na_2CO_3 and extracted in a chloroform/ethanol mixture (80:20). The extract was analysed by means of thin layer chromatography (tlc) and HPLC systems. The tlc system consisted of silica gel G_{254} as the stationary phase and ethanol–chloroform–ammonia (50:49:1) as mobile phase. The HPLC consisted of a Pye Unicam RP 18 column (10 μm; 12.5 cm long), and a mobile phase of methanol–0.005 M KH_2PO_4 (pH 4) (20:80, v/v). The flow rate was 2 ml/min and detection was by UV absorption at 330 nm using a Pye Unicam SP3-800 spectrophotometer. The internal standard used was 2-methyl-5-nitroimidazole. The UV spectra of the synthesized tinidazole *N*-oxide and the standard tinidazole in ethanol were recorded. The ethanol solution of the *N*-oxide product was treated with sulphur dioxide (generated by the addition of concentrated HCl to $Na_2S_2O_5$) and the UV absorbance of the resulting product recorded.

In-vitro study

Liver microsomal preparations were made as follows: to 2 ml of the ice-cold liver homogenate (10,000 *g*) were added the following: 2 ml phosphate buffer (pH 7.4); 1 ml cofactor solution (nicotinamide, 0.6 M, 1 ml; magnesium chloride, 0.01 M, 2 ml; glucose-6-phosphate, 60 mg; NADP 34 mg; and water to a total volume of 10 ml) and 1 ml of substrate (1 mg/ml) in a 25 ml conical flask. This was replicated 10 times and divided into two sets of five. These were incubated in a rocking water bath at 37°C for 30 and 60 min respectively, after which they were immediately placed in an ice-cold bath. The contents of the flask were then extracted with chloroform–ethanol, concentrated and examined on the tlc, UV and HPLC systems described. Tinidazole *N*-oxide was obtained by preparative tlc and characterized by UV and HPLC.

In-vivo study

Three healthy humans (age range 21–25, average weight 67 kg) who had fasted overnight, were given tinidazole (4 × 500 mg) orally. None of the subjects had been on any medication in the previous 2 months. Blood and urine samples were taken at intervals of 1, 4 and 8 h and then 1 week after medication. The blood and urine samples were analysed im-

mediately after collection by tlc, and according to the modified HPLC method previously reported [6].

Serum analysis. One part of serum (2 ml) was mixed with one part of 5% (v/v) perchloric acid and the mixture stirred rapidly for 1 min with a Vortex blender. The mixture was then centrifuged for 5 min (1000 *g*), the clear supernatant filtered (Whatman No. 4 filter paper) and 20 µl of filtrate injected onto the HPLC column. The flow rate was 2 ml/min and the absorbance detector was set at 340 nm. The serum sample, after filtration, was treated with sulphur dioxide and the resulting solution was assayed chromatographically.

Urine analysis. One part of urine (4 ml) was mixed with one part of 5% perchloric acid (v/v) and the solution stirred rapidly for 30 sec. Chloroform (8 ml) was added to the mixture which was again stirred rapidly for a further 20 sec and then centrifuged for 5 min (1000 *g*). The upper phase was separated, filtered through Whatman No. 4 filter paper and spiked with internal standard. Twenty microlitres of the resultant mixture was injected into the HPLC column. The eluting solvent system was the same as in serum analysis. The sample solution was also treated with sulphur dioxide and rechromatographed.

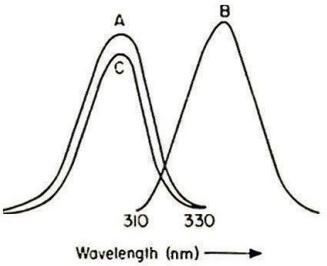

Fig. 2. The UV spectra of: (A) tinidazole; (B) tinidazole *N*-oxide; (C) tinidazole; obtained after treatment of tinidazole *N*-oxide with sulphur dioxide. All solutions were made in methanol.

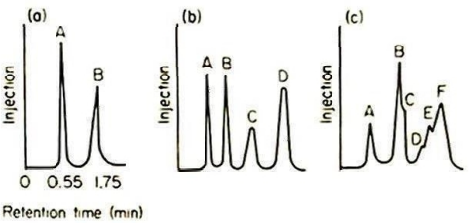

Fig. 3. High performance liquid chromatograms of: (a) in-vitro incubation extract: A = metabolic *N*-oxide; B = internal standard; (b) blood extracts: A = tinidazole *N*-oxide; B = internal standard; C and D = unidentified metabolites; and (c) urine extracts: A = tinidazole *N*-oxide; B = internal standard; C, D, E and F = unidentified metabolites. See Materials and methods for HPLC details.

Results and discussion

Tinidazole *N*-oxide showed a characteristic single homogeneous spot on tlc (R_f 0.6). There are two nitrogen centres in the imidazole ring and in a previous metabolic study involving metronidazole we had shown that the preferable centre of reaction is *N*-3 which is a more basic centre than the *N*-1, and also that *N*-3 has less steric hindrance than *N*-1 [1]. The ultraviolet spectra of the *N*-3 oxide and the parent tinidazole in ethanol showed that the tinidazole *N*-oxide absorbance had undergone a bathochromic shift (λmax 330 nm) relative to tinidazole absorbance (λmax 310 nm) (Fig. 2). The *N*-oxide was authenticated further using a mild and selective reducing agent, sulphur dioxide (SO_2) [8]; HPLC analysis of the *N*-oxide product showed a characteristic retention time of 0.55 min (Fig. 3), the authenticity of the peak was proven by subjecting the solution of the product to SO_2 treatment, causing the disappearance of the characteristic peak.

The synthetic and biologically produced *N*-oxides demonstrated similar physicochemical characteristics, i.e. R_f 0.6, on the same tlc systems, silica gel G_{254} and ethanol–chloroform–ammonia (50:49:1). Both products gave identical UV absorption spectra and also underwent the same chemical reduction process. It was obvious that the numerous peaks obtained during in-vivo HPLC analyses of serum and urine samples were those of many metabolites of tinidazole. It is most probable that the metabolite with the characteristic HPLC retention time of 0.55 min is tinidazole *N*-oxide. The identity of this metabolite was further established by treating the serum and urine extracts with SO_2. The resulting chroma-

togram showed disappearance of this characteristic peak. These findings tend to support the fact that tinidazole is metabolized by oxidative processes (mixed function oxidases) with fortified hepatic fractions (*in vitro*) and also *in vivo* when administered to man.

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