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ORIGINAL

Nitazoxanide: pharmacokinetics and metabolism in man

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Key words
nitazoxanide – tizoxanide – metabolism – antiprotozoal – radiocarbon – ADME

Abstract. Objectives: Nitazoxanide (N), a new broad-spectrum parasiticidal agent, is rapidly deacetylated to tizoxanide (T). The objective of the study was to determine if metabolites other than T are present in the plasma and excreted after single dose oral administration of radiocarbon-labelled N in healthy subjects. Methods: Six healthy volunteers received a single 500 mg oral dose of N labelled with 2.92 MBq radiocarbon. The radioactivity in blood, plasma, urine, feces and expired air was monitored at scheduled intervals for up to 10 days. Selected samples were assayed by HPLC for T and submitted to metabolite identification by mass spectrometry. In vitro experiments were also conducted (incubation with animal and human microsomes, deacetylation kinetics). Plasma and bile samples obtained in a patient treated with N for sporozoal infection were also assayed for T. Results: Elimination of radiocarbon occurred both in the urine (31.5% of the dose on average) and in the feces (66.2% on average). T and T-glucuronide contributed 15% of total urine radioactivity. N was found to deacetylate extremely rapidly to T in plasma (half-life of about 6 minutes at 37° C) as well as in presence of liver microsomes. T was the only species obtained by incubation with human microsomes while rat microsomes yielded hydroxylated T in addition. The main species identified in human plasma, urine and bile was T-glucuronide, the identification of which was confirmed by comparison with an authentic sample. No species other than T was detected in feces, indicating intensive intestinal deconjugation, while radioactivity and absorbance detectors showed largely unresolved clusters.

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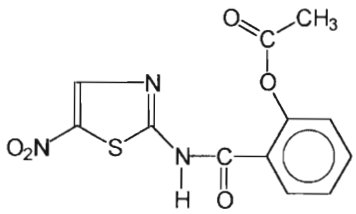
Introduction

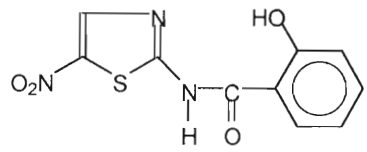
Nitazoxanide (N) is a nitrothiazole benzamide compound [Rossignol and Cavier 1976], effective against a wide variety of parasites, bacteria and fungi infecting animals or humans. In addition to numerous preclinical studies [Blagburn et al. 1998, Cavier and Rossignol 1982, Didier et al. 1998, Dubreuil et al. 1996, Euzeby et al. 1980, Megraud et al. 1998, Theodos et al. 1998], N has shown clinical efficacy in the treatment of infections by many parasites [Abaza et al. 1998, Romero Cabello et al. 1997, Rossignol and Maisonneuve 1984, Rossignol et al. 1998], including Cryptosporidium parvum in patients with AIDS [Doumbo et al. 1997, Rossignol et al. 1998]. The drug has shown efficacy in vitro against protozoa such as Trichomonas vaginalis and Entamoeba histolytica; in vivo against Cryptosporidium parvum as well as against nematodes such as Syphacia obvelata, Uncinaria stenocephala and Trichuris vulpis; cestodes such as Dipylidium caninum, Taenia pisiformis and Hymenolepis nana. N is also effective in vitro against a broad range of Gram-positive and Gram-negative anaerobic bacteria and against aerobic Gram-positive bacteria, as well as against dermatophytes.

The pharmacokinetics of N have been studied after single dose oral administration in healthy subjects [Stockis et al. 1996], showing that no parent compound could be found either in plasma or in urine. In plasma, the only relevant species that could be found was deacetyl-nitazoxanide (tizoxanide, T), whereas in enzymatically deconjugated urine, T accounted for 5.4% of the dose, salicylurate for 2.8%, and aminonitrothiazole for 0.1% (Figure 1).

The objective of the study was to determine if metabolites other than T are present in the plasma and excreted after single-dose oral



nitazoxanide



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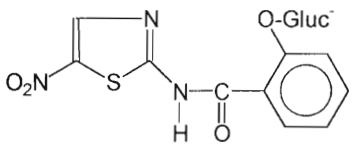

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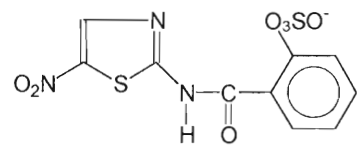

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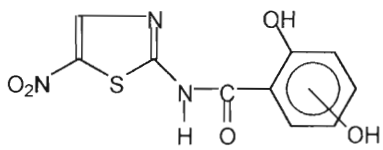
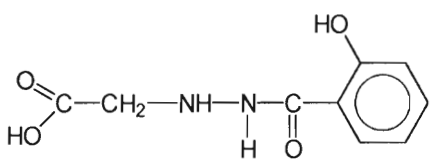
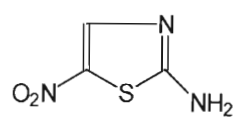


hydroxy tizoxanide



salicyluric acid



aminonitrothiazole

Figure 1. Nitazoxanide and metabolites (main circulating species are in bold).

administration of radiocarbon-labelled N in healthy subjects.

Subjects, materials and methods

The study was conducted in accordance with the guidelines set by the World Medical Assembly (Declaration of Helsinki, last amended in Hong Kong in 1989) as well as with the recommendations of the World Health Organization [WHO 1977] and of the International Commission on Radiological Protection [ICRP 1993] regarding the use of ionizing radiation on human beings for medical research. The protocol of the study and the informed consent form were both approved

by the Medical Ethics Committee of the public hospitals of Antwerp (Belgium) on 8 May 1996. Six healthy male subjects, aged 24 – 50 years, volunteered to take part in the study and signed an informed consent form detailing the procedures and constraints as well as the risks involved. Prior to enrolment, each subject underwent a thorough medical examination involving detailed history, physical examination, 12-lead ECG and laboratory tests (incling hematology, serum chemistry and urinalysis).

The subjects received, after an overnight fast, a single oral 500 mg dose (1.63 mmol) of phenyl-ring-UL-^{14}C-N (custom-prepared by Sigma Chemical Company) containing 2.92 MBq radiocarbon. Blood samples were obtained by venipuncture at scheduled intervals for up to 10 days. All urine and feces were collected during the same period. Several samples of expired carbon dioxide were collected over 24 hours post dose. All samples were assayed for radiocarbon by liquid scintillation, either directly (by mixing with Ready Safe, Beckman, for plasma and urine, or with Hionic-Fluor, Packard, for expired CO_2) or after combustion in a Sample Oxidizer, Packard (for whole blood and feces homogenates). Selected samples were also assayed for T by HPLC [Stockis et al. 1996]. Concentration in blood cells was derived from concentrations in whole blood and in plasma as $C_{bc} = [C_b - C_p \times (1 - Ht)]/Ht$, where Ht stands for hematocrit (determined once daily).

Samples of plasma, urine and feces were also submitted to mass fragmentometry, eventually after HPLC (reverse-phase Nucleosil 5C18 column, elution by a mixture of 10 mM aqueous formic acid and acetonitrile, linear gradient from 10% to 90% acetonitrile), using a VG Quattro II tandem mass spectrometer, Fisons, and collecting negative ions from 100 to 650 Da (full scan mass spectrum in 1.4 s). Some urine samples were submitted to enzymatic deconjugation prior to analysis: 2 ml of urine was mixed with 20 µl of 100 U/ml β-glucuronidase and 5 µl of 25 U/ml arylsulfatase from Helix Pomatia (both from Boehringer Mannheim) and 0.2 ml of 1 M pH 5.5 acetate buffer, and incubated a 37° C during 3 hours. After acidification with 0.2 ml of 1 M formic acid and centrifugation, aliquots of 0.1 ml were injected into the HPLC system.

^{14}C-N was incubated with hepatic microsomes from rats (either normal or after induction of cytochrome-P450s by methylcholanthrene, phenobarbital or dexamethasone), from Beagle dogs, from Cynomolgus baboons, and finally from human livers. Normal and induced animal microsomes were obtained from Iffa Credo; human microsomes were prepared from liver specimens (University hospital of Liège, Belgium, and France Transplant) according to a standard procedure [Kremers et al. 1981]. The microsomal suspension (corresponding to about 0.4 mg protein) was incubated at 37° C with 50 µg of N in 1 ml of cofactors solution (composition per ml: 0.4 mg NAD, 0.4 mg NADP, 0.75 mg G-6-P, 1 mg $MgCl_2 \times 6H_2O$, 2 µl of 350 U/ml G-6-P dehydrogenase and 0.1 ml of 0.1 M pH 7.4 TRIS buffer). Two control samples, one containing only the cofactors and the other the cofactors and heat-inactivated rat microsomes, were also added. Incubation was terminated after 0, 15, 30, 60 or 120 minutes (0 and 120 minutes only for human microsomes and controls) by adding 0.1 ml of 2 M formic acid and 0.55 ml of acetonitrile. The mixture was then centrifuged and the supernatant was injected on the HPLC column.

In addition, plasma and bile samples were obtained in a patient being treated at the Tübingen Institute of Tropical Medicine with N 1000 mg orally for Isospora belli infection of the biliary tract and small intestine. These samples were collected 1 h, 2 h, 4 h and 8 h after the first dose; a second dose was given 9 h after the first one and a plasma and a bile sample were obtained 3.5 h later. The samples were assayed for T by HPLC, before and after enzymatic deconjugation.

Results

Tolerability

The single oral dose of 500 mg N was well tolerated. One of the subjects dropped out of the study for personal reasons after 86 hours, before complete excretion of the radioactive label (86.8% of the dose was recovered). In the other subjects, the excretion balance averaged 97.7% (range: 92.9 – 100%). Assuming whole body irradiation, the effective dose

Table 1. Individual and mean (± SD) pharmacokinetic parameters for total ^{14}C and tizoxanide by HPLC.

Parameter	Subject No.	1	2	3	4	5*	6	Mean	SD
C_{max} (nmol-eq×g^{-1})	^{14}C	17.0	15.8	11.1	24.3	28.4	9.9	17.8	7.3
	T by HPLC	3.35	4.34	3.85	5.55	8.78	2.45	4.71	2.22
t_{max} (h)	^{14}C	5	4	2	5	4	3		
	T by HPLC	2	4	2	4	3	3		
AUC (nmol-eq×h×g^{-1})	^{14}C (0-240 h)	389	306	185	478		285	329	111
	^{14}C (0-10 h)	95	81	61	135	151	55	96	39
	T by HPLC (0-10 h)	13.2	10.6	10.3	19.5	26.0	7.8	14.6	6.9
X_U (% of dose)	^{14}C	33.2	35.4	27.7	30.6	*35.2*	30.7	31.5	2.9
	T by HPLC	4.8	4.2	2.3	7.3	7.6	2.8	4.8	1.4
X_F (% of dose)	^{14}C	66.2	63.2	70.1	69.4	*51.6*	62.2	66.2	3.6
X_{U+F} (% of dose)	^{14}C	99.4	98.6	97.8	100.0	*86.8*	92.9	97.7	2.8

* = Subject No. 5 dropped out after 86 hours: italicized data are not included in calculation of mean and SD

ranged from 25 to 230 µSv for all 6 subjects, corresponding to a category where the level of risk ranges from trivial to minor [ICRP 1993].

Plasma and blood levels

In plasma, the radioactivity rose to maximum values (C_{max}) which, expressed in units of nmol N-equivalent per ml, ranged from 9.9 to 28.4. The time to peak (t_{max}) occurred 2 to 5 hours after intake. The area under the concentration-time curve (AUC), computed by the linear trapezoidal rule, from 0 to 240 hours, ranged from 185 to 478 nmol N-eq×h×ml^{-1} in the subjects who completed the study (Table 1). It appears from the measurements in whole blood that the label was not bound to blood cells: in most samples, the calculated specific radioactivity was below the quantification limit.

No unchanged N was found in any sample. The plasma concentration of T, measured by HPLC, contributed for about 27% to the radioactivity at C_{max}, with values in the 2.5 – 8.8 nmol/ml range, and for about 15% to the AUC of total radiocarbon measured over 10 hours (Table 1). At later times, T concentration was always below the limit of quantitation (< 0.075 nmol/ml), whereas ^{14}C remained well above its limit of quantitation (corresponding to 0.25 nmol N-eq/ml) for about 10 days (Table 1).

Excretion

The radioactivity excreted as carbon dioxide was negligible. Elimination of radiocarbon occurred both in the urine (X_U = 31.5% of the dose on average) and in the feces (X_F = 66.2% on average) in the subjects who completed the study. Since the collection of excreta was stopped after subject No. 5 left the study, his excretion balance was lower with 35.2% in the urine and 51.6% in the feces. The urinary excretion of T, measured after enzymatic hydrolysis of the samples, reached about 5% of the dose and contributed to about 15% of total urine radioactivity (Table 1).

Chemical stability of N

The absence of the parent compound N in plasma has been ascribed to the action of plasma esterases [Stockis et al. 1996]. The stability of N was examined in various media. In organic solvents like acetonitrile, dimethylsulfoxide and acetone, N solutions were stable when stored at 4° C for at least 8 days. In aqueous buffers, however, N hydrolyzed readily into T in a temperature- and pH-dependent way. The reaction seemed to follow first-order kinetics so that an apparent half-life could be measured. At 4° C, the half-life shortened from about 50 days at pH 5 to about 60 hours at pH 7 – 8. At 25° C, it decreased from about 86 hours at pH 5 to 13 hours at pH 7 – 8. At 37° C, it fell from

Table 2. In vitro biotransformation by several types of hepatic microsomal preparations.

liver microsomes	incubation time (min)	% ^{14}C extracted	N	T	hydroxy-T (in % of extracted ^{14}C)
none (cofactors alone)	120	98	65	35	0
rat, inactivated	120	72	62	34	0
rat, normal	120	82	0	77	23
rat, phenobarbital	120	86	0	64	23
rat, dexamethasone	120	80	0	65	29
rat, methylcholanthrene	0	94	87	13	0
	15	67	7	93	0
	30	81	0	87	13
	60	86	0	59	39
	120	94	0	36	59
dog	120	84	0	100	0
baboon	120	75	0	94	< 6
human (n = 5)	120	66	0	100	0

33 hours at pH 5 to 3 hours at pH 8. In fresh human plasma, the apparent half-life dropped to 50 minutes at 4° C, 9 minutes at 25° C, and about 6 minutes at 37° C. The same values were obtained when N was incubated with plasma diluted tenfold. The stability of N was also tested in mixtures of organic solvents and aqueous buffers to determine their suitability as mobile phase in HPLC. The best mixture was made of 20% pH 3 formate buffer and 80% acetonitrile.

In vitro metabolism of N

When incubated without microsomes or with heat-inactivated microsomes for 2 hours, about 35% of N deacetylated to T (Table 2). In the presence of microsomes, deacetylation was much faster, so that only traces of N remained after 15 minutes of incubation. With microsomes from dogs and from humans (5 distinct samples), T accounted for the entire extracted radioactivity. However, it should be stressed that the recovery was incomplete, since up to a third of the added radioactivity remained in the centrifugation pellet. Another compound contributed to the extracted radioactivity with baboon and rat microsomes, for a very small part with the former but for a quite substantial part with rat microsomes. T appeared first, almost accounting for the entire extracted radioactivity during the first 30 minutes of incubation, and then decreased over time while the other compound appeared gradually. The time profiles in normal rat microsomes where essentially identical to those in dexamethasone- and phenobarbital-induced microsomes, while in those from methylcholanthrene pre-treated animals the unknown compound became the predominant species after 120 minutes of incubation (Table 2). This metabolite eluted faster than N and T and has been submitted to analysis by mass spectrometry. A strong ion signal at m/z 280 indicated a molecular mass of 281, i.e. smaller than that of N (307), but larger than that of T (265) by 16 mass units. The same shift of 16 units was also observed for two characteristic fragments of T, at m/z 217 and 190, which were found in the metabolite at m/z 233 and 206, respectively. Two other characteristic fragments of T, at m/z 144 and 114, which can be ascribed to the heterocyclic moiety (the amino-nitrothiazole fragment) before and after loss of the NO group, respectively, were found unchanged in the metabolite. It was tentatively concluded, therefore, that the metabolite, found only with microsomes from the rat (and to a much smaller extent with those from the baboon) might be considered as T hydroxylated on the phenyl ring (Table 2).

Mass spectrum analysis of urinary samples

The chromatograms of the early urine samples from subjects treated with ^{14}C-N showed a single major radioactive and light-absorbing component eluting faster than both N and T. The mass spectrum showed a molecular ion signal at m/z 440, a very strong one at m/z 264 (similar to the molecular ion of T), as well as the 217, 190 and 144 fragments characteristic of T. The difference in molecular mass between T and the unknown compound, termed metabolite I, i.e. 176, suggested the presence of a glucuronide of T.

Other radioactive material eluted still faster but was not separated properly. By modifying the chromatographic conditions (shorter gradient, slower flow rate, less formic acid), it was possible to separate several light-absorbing peaks and to submit the collected fractions to mass spectrometry. One of them, called metabolite II, showed a molecu-

lar ion at m/z 344 and a very strong signal at m/z 264. The difference in molecular mass between this metabolite and T, i.e. 80, suggested the existence of a sulfoconjugate of T. Another one, called metabolite III, showed a molecular ion at m/z 360 (i.e. 16 mass units above that of metabolite II) and a strong signal at m/z 280 (i.e. 16 mass units above that of T). These findings were suggestive of the presence of a sulfoconjugate of hydroxylated T.

Mass spectrum analysis of plasma samples

Plasma samples from subjects treated with ^{14}C-N were extracted by mixing with 1.5 volumes of acetonitrile, discarding the precipitate by centrifugation, evaporating the supernatant to dryness and reconstituting with a mixture of water and acetonitrile (2/1 v/v). The radioactivity of these extracts was too low for radiochromatographic detection, but light-absorbing components eluting as T and metabolite I were found. In the earliest samples, T was the major component, but the peak of metabolite I became dominant after about 3 hours.

Mass spectrum analysis of fecal samples

Fecal samples from subjects treated with ^{14}C-N were lyophilized and the dry powder was extracted with mixtures of water and acetonitrile. After centrifugation, the extracts were submitted to HPLC. Both the radioactivity and light absorbance detectors showed largely unresolved clusters. With the mass detector tuned on the relevant ions for N, T, and metabolites I, II and III, no species other than T could be detected.

Identification of metabolite I

If metabolite I, found as the main component in early urine samples from subjects treated with ^{14}C-N, were indeed T glucuronide, then enzymatic hydrolysis should free a sizeable concentration of T. When the samples were incubated with β-glucuronidase and arylsulfatase, only a small fraction of the radioactivity was found in the supernatant after centrifugation. More radioactivity could, however, be extracted progressively from the pellet with acetonitrile. The main component in all extracts had the analytical characteristics of T.

In order to confirm the identity of T glucuronide, an authentic sample was prepared by reacting benzyl salicylate with the methyl ester of α-bromo-tri-O-acetyl-glucuronic acid [Rossignol and Stachulski 1999]. The expected acetylglucuronide was debenzylated and amidated with 2-amino-5-nitrothiazole to yield the methyl ester of acetyl-glucuronyl T, which was finally hydrolyzed into the sodium salt of T glucuronide. This compound showed the same analytical characteristics (HPLC, fragmentometry), as metabolite I. It is also of interest to note that the sodium salt is sparingly soluble in water. Unfortunately, it has not been possible to obtain the sulfoconjugate of T for comparison with metabolite II.

Determination of T glucuronide in plasma

After oral administration of radiocarbon-labelled N, the concentration of T in plasma contributed to 27% on average of the radioactivity at C_{max}, to a mean 15% of the AUC over 10 hours, and to much less when considering the AUC of radiocarbon over 10 days. Since T glucuronide was found as the main metabolite in urine, and was also shown to be present in plasma samples, it was hypothesized that it could be a main circulating metabolite. Therefore, the kinetics of its hydrolysis by β-glucuronidase (Sigma, type HP2) in acetate buffer at 37° C and the stability of T in the incubation medium were measured over several hours in order to define the best method for assaying both substances. Blank plasma samples were spiked with either T or T glucuronide, incubated with the enzyme for various time periods, and assayed for T. Deconjugation was already complete after 0.5 h and no degradation occurred until 2 hours of incubation. During that time, the samples spiked with T did no show any change in concentration. A one-hour hydrolysis was thus selected.

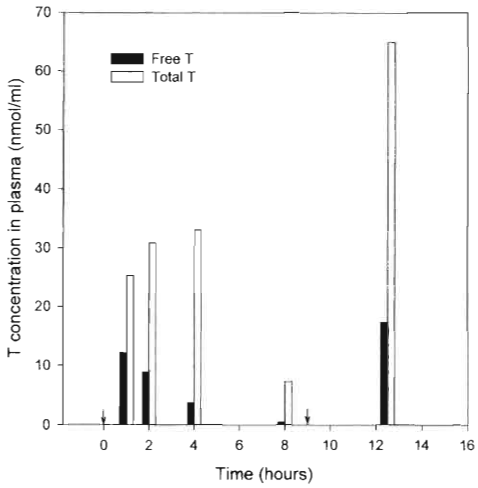


Figure 2. Concentration of tizoxanide in plasma assayed before (filled bars) and after enzymatic deconjugation (open bars) in a patient given twice (arrows) 1000 mg nitazoxanide.

These enzymatic hydrolysis conditions were applied to plasma samples obtained from Dr. Overkamp, Tübingen, Germany, who treated a patient with N. The results (Figure 2) show that the concentration of conjugated T was always higher, up to 15 times, than that of free T. The same method was used to assay bile samples from the same patient: the concentration of conjugated T, ranging from 43 to 770 nmol/ml, was 700 to 2100 times larger than that of free T. The bile to plasma ratio was about 10. Figure 2 also pointed to a possibly slower clearance of conjugated T, compared to T.

Discussion and conclusion

When N is given orally, no parent compound can be found in any type of sample, i.e. plasma, urine or feces. It was shown, indeed, that N hydrolyses spontaneously into T in aqueous media, and much more rapidly in the presence of plasma. The rate of deacetylation is pH- and temperature-dependent, and the half-life of N added to plasma is about 6 minutes at 37° C. Therefore, no unchanged N could ever remain in plasma for any useful time.

In the presence of liver microsomes, N deacetylates also readily to T. No other metabolite could be observed with canine and human microsomes. With rat microsomes, however, a molecular species was discovered with mass spectrometric characteristics of a phenyl-hydroxylated T. Thus, the amide bond linking the salicyl moiety of T to the thiazole ring seems largely resistant to enzymatic cleavage, in accordance with the very small amount of aminonitrothiazole found in the urine [Stockis et al. 1996].

Although a sulfoconjugate of T and a sulfoconjugate of hydroxylated T might have been detected in the urine of subjects treated with 500 mg N, it appears that the glucuro-conjugate of T is the main metabolite in plasma, urine and bile. This metabolite seems to be cleared more slowly from the plasma than the free form, perhaps because of its poor solubility. It also seems to be entirely hydrolyzed in the intestine, since only non-conjugated T could be detected in feces. The observation of a cluster of unresolved peaks in the radiochromatograms of fecal samples also points to further intensive biotransformation.

In conclusion, significant circulating species after oral administration of N seem to be limited to T and T glucuronide. Since authentic T glucuronide has now been synthesized and has shown to be effective against both parasites and bacteria [Rossignol and Stachulski 1999], it should be assayed directly (i.e. without prior conversion to free T) in plasma, urine and feces in order to estimate its contribution to the disposition of nitazoxanide.

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