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Pharmacokinetics of Amonafide in dogs

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Summary. Amonafide, one of a series of imide derivatives of 1,8-naphthalic acid synthesized by Brana et al. [2] has shown significant antitumor activity against a variety of experimental tumors, including L1210 leukemia and P388 leukemia. Along with the clinical trial at our institute, we have studied the disposition of Amonafide in dogs by HPLC and fluorometry. Six dogs received Amonafide i.v. at 5 mg/kg (100 mg/m^2) over 15 min; three were sacrificed at 6 h, and three at 24 h. The initial plasma $t_{1/2}$ of Amonafide was 2.4 ± 0.4 min, the intermediate $t_{1/2}$, 26.8 ± 3.7 min, and the terminal $t_{1/2}$, 21.7 ± 4.0 h. The peak plasma concentration achieved was 6.3 ± 1.7 μg/ml. The average apparent volume of distribution was 12.84 ± 0.54 l/kg, and the total clearance was 0.56 ± 0.16 l/kg/h. In 24 h, 9.5% ± 0.2% of the administered dose was excreted in the urine as the parent drug, and 7.4% ± 1.4% in the bile in 6 h. Amonafide penetrated the CSF readily and achieved the highest concentration 20–25 min after administration, which was 30% of the concurrent plasma level. Amonafide underwent extensive metabolism to at least three major metabolites and two or more minor metabolites. The α and β plasma $t_{1/2}$ of the major metabolite, an *N*-oxide derivative, were 24.8 min and 28.6 h, respectively. The 24-h cumulative urinary excretion was 1.4% of the injected dose, and the cumulative biliary excretion was 16.7% in 6 h. At autopsy 6 h after dosing, the liver contained the highest percentage (0.23% of administered dose) of unchanged Amonafide, followed by the stomach (0.11%), lung (0.04%), kidney (0.04%), and pancreas (0.03%). The rest of the major organs retained less than 0.02% of the Amonafide dose. One day after dosing, no detectable amount of Amonafide was found in any of these tissues, indicating that Amonafide appears to be extensively metabolized and not significantly retained in the dog.

Introduction

1-*H*-Benz[de]isoquinoline-1,3(2*H*)-dione, 5-amino-2-[2-(dimethylamino)ethyl]-(Benzisoquinolinedione, NSC-308847, Nafidimide or Amonafide) is one of a series of imide derivatives of 3-nitro-1,8-naphthalic acid that were synthesized by Brana and associates from Spain [2]. The rationale for the synthesis of this compound was to combine into a single molecule the structural entities believed to be responsible for the antitumor activity in aristolochic acid: cycloheximide, tilorone, and 1-(morpholinomethyl)-4-phtalimido piperidin-2,6-dione. The 2-substituted dialkylamino alkyl derivatives of 5-nitrobenz-(de) isoquinoline-1,3-dione demonstrated antitumor effects against HeLa and KB cell lines in vitro, Ehrlich's carcinoma in mice, and Walker and Yoshida tumors in rats. Because of these growth inhibitory effects, additional congeners were synthesized, including Benzisoquinolinedione, which also inhibited the growth of HeLa and KB cells in vitro [3]. The Benzisoquinolinediones appear to function as DNA intercalating agents. Recent studies from our laboratory with Amonafide have demonstrated that this compound stabilized DNA to thermal denaturation, produced protein-associated DNA single-strand breaks, and blocked both nucleic acid and protein synthesis [1].

Amonafide was selected for development to clinical trials based on its activity against the i.p. implanted L1210 leukemia and P388 leukemia (Investigational Drug Branch, NCI, unpublished data). The drug produced a significant increase in the life span of tumor-bearing animals over a two-fold dose range. The best antitumor effect was obtained with the intermittent multiple-dose schedule, compared to a single-dose administration [3]. In addition to its activity against murine leukemia, Amonafide also demonstrated activity against M5076 sarcoma and B_{16} melanoma. Antitumor activity was observed even when the tumor implant site and drug injection site were not the same.

Amonafide was evaluated in preclinical toxicology studies using CDF_1 mice and beagles [3]. Animals were injected i.v. on a single-dose or on five-dose daily regimens. In the mouse lethality studies, all the toxic signs and death occurred on day 1 after a single dose. Toxic signs consisted of labored breathing, struggling, and exophthalmus; additional signs of toxicity consisted of loss of locomotor ability, decreased activity, and rough hair coats. All surviving animals were normal within a few hours. There were no differences in the tolerance to the dose levels tested. On the five-dose daily schedule, death occurred on days 1 and 10 and the toxic signs included labored breathing, exophthalmus, hyperextension of limbs, loss of locomotor ability, struggling, hunched posture, increased irritability, squinted eyes, and marked prostration. Furthermore, the animals were observed to have swollen and necrotic tails, swollen faces with closed eyes, and ataxia. Most deaths oc-

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curred on days 6 and 10, although three animals died on days 2–5. By day 10, the mice had either died or recovered from all symptoms except the tail lesions, which persisted to termination on day 33.

Because the starting dose of Amonafide for clinical trials was chosen on the basis of dog toxicology data, we have studied the preclinical pharmacology of this agent in dogs in conjunction with phase 1 trials of Amonafide.

Materials and methods

Drugs. HPLC-grade acetonitrile and methanol were purchased from Burdick Jackson Laboratories (Muskegon, Mich, USA). Amonafide was kindly supplied by the Development Therapeutics Program, Division of Cancer Treatment, National Institutes of Health (Bethesda, Md, USA). Stock solutions of Amonafide were prepared by adding 4 ml sterile water to 100 mg lyophilized drug, to yield a final Amonafide concentration of 25 mg/ml. All other chemicals were of analytical grade and were obtained from regular commercial suppliers.

Dogs. Mongrel dogs of either sex, weighing 15–20 kg, were lightly anesthetized with pentobarbital. Amonafide (5 mg/kg) was administered over 10 min through the femoral vein. At predetermined intervals, 10-ml blood samples were collected from the opposite femoral vein, using sodium heparin as the anticoagulant. The blood samples were centrifuged at 12,000 *g* for 10 min at 25° C to obtain plasma. Amonafide concentration in plasma was determined as described below.

Urine was sampled with a Foley catheter. The bladder was flushed with 0.9% NaCl solution after each collection, and the washings were combined with the appropriate urine sample. CSF was sampled by cisternal puncture, and bile was sampled by cannulation of the common bile duct.

The animals were killed with an overdose of anesthetic agent 6 or 24 h after drug administration. The major organs were dissected, freed of fatty tissues, and weighed. Amonafide and metabolites of each organ were determined as outlined below.

Chromatography. Amonafide was quantitated by a sensitive and specific HPLC method developed in our laboratory [5]. Briefly, the drug was eluted by an isocratic HPLC method using a mobile phase of 0.02 *M* acetate buffer (pH 4.0) in 15% acetonitrile on a 4 mm × 30 cm μBondapak C_{18} reverse-phase column (Waters Associates, Milford, Mass, USA).

All analyses were carried out on a Waters Associates high-pressure liquid chromatograph (Milford, Mass, USA) equipped with a Model M-6000A pump, U6K injector, and Schoeffel Model SF-970 fluorescent detector (Kratos Analytical Company, Westwood, NJ, USA), with the excitation wavelength set at 350 nm and emission set at 550 nm.

Sample preparation and extraction. A C_{18} Sep-Pak (Waters Associates, Milford, Mass, USA) was used as a minichromatographic column to prepare the plasma, urine, bile, and CSF samples according to a method previously published [5].

To extract Amonafide from tissues, a 10-g specimen was washed with normal saline, blotted dry, and minced in 10 ml 0.02 *M* acetate buffer, pH 4.0. The mixture was homogenized with a model PT-10 polytron tissue homogenizer and centrifuged at 12,000 *g* for 10 min. The supernatant was then collected and extracted as described above for the biological fluids.

Recovery of the C_{18} Sep-Pak extraction procedure for the parent drug was 87% for plasma, 80% for urine, and 75%–80% for tissue [5].

Pharmacokinetic computations. Experimental results were subjected to computerized sample, nonlinear regression analysis. Best-fit criteria were based on correlation coefficient determination and the *F*-test. Pharmacokinetic parameters were computed by the PCNONLIN program (Statistical Consultants, Inc., Lexington, Ky, USA).

Isolation of urinary metabolite. Urine was extracted twice with an equal volume of 1:1 (v/v) methanol:chloroform. The combined organic phases were evaporated to dryness by a flash evaporator. The residue was reconstituted with 2–5 ml methanol:chloroform mixture, an aliquot of which was applied to silica gel GF TLC plates (Brinkman). The plates were developed using chloroform and methanol (1:1, v/v). The various fluorescent areas of parent drug and metabolites were scraped from the plates and eluted with methanol, and the eluents were brought to complete dryness with nitrogen. The identity and purity of the isolated drug and metabolites were checked by HPLC and then subjected to thermospray liquid chromatography-mass spectrometry (LC/MS) analysis as described below.

Thermospray LC/MS metabolite analysis. Urine extracts were dissolved in 1 ml 0.1 *M* ammonium acetate. Aliquots (20 μl) of these samples were injected onto a μBondapak CN HPLC column (Waters Associates, Milford, Mass, USA) and chromatographed, using a mobile phase of 0.1 *M* ammonium acetate:acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min. A Perkin Elmer series 4 HPLC system was interfaced to a Hewlett Packard model 5982 mass spectrometer using a Vestec Thermospray LC/MS interface (Vestec Corp., Houston, Tex, USA). The thermospray interface was operated without any means of external ionization (Filament "Off" mode). Optimal thermospray temperatures for this analysis were determined at 175° C for the vaporizer probe tip and 305° C for the thermospray ion source. A complete description of the LC-MS procedure for the determination of Amonafide and its associated metabolites has been published elsewhere [4].

Results and discussion

In six dogs, after i.v. administration (5 mg/kg or 100 mg/m^2), of Amonafide, the disappearance of the parent drug from the plasma followed a triphasic pattern (Fig. 1), with an average initial $t_{1/2}$ of 2.4 ± 0.4 min, an intermediate $t_{1/2}$ of 26.8 ± 3.7 min, and a terminal $t_{1/2}$ of 21.7 ± 4.0 h, as estimated by NONLIN regression analysis (Table 1). The highest observed drug concentration in the plasma after the end of injection was 6.3 ± 1.7 μg/ml. Also included in Table 1 are the values of the other pharmacokinetic parameters. The average apparent volume of distribution was 12.84 ± 0.54 l/kg, and the total clearance was 0.56 ± 0.16 l/kg/h. In 24 h, 9.5% ± 0.2% of the administered doses was excreted in the urine (Fig. 2), whereas 7.4% ± 1.47% was ex-

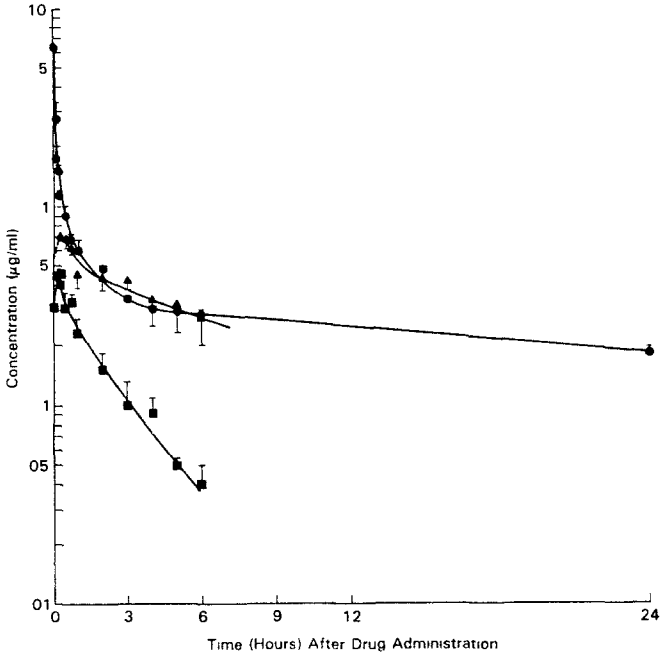

Fig. 1. Plasma and CSF levels of Amonafide and *N*-oxide metabolite. –●–, plasma decay of Amonafide; –▲–, plasma decay of *N*-oxide metabolite; –■–, concentration of Amonafide in CSF. Data are presented as mean ± SD from six dogs

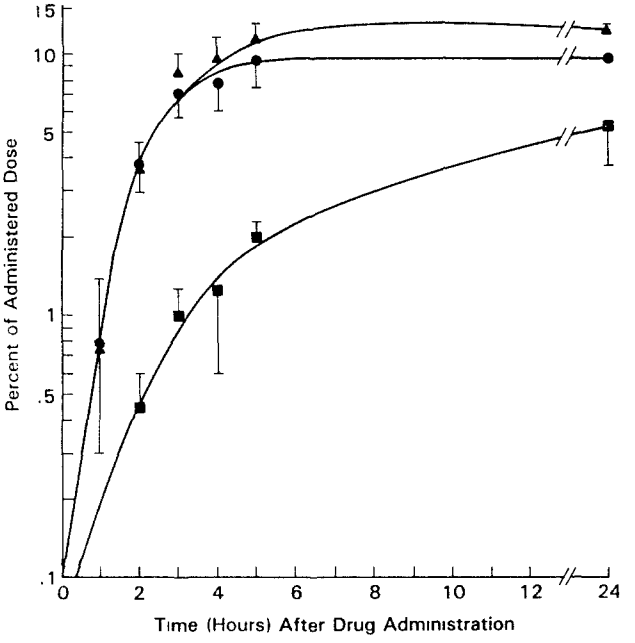

Fig. 2. Cumulative urinary excretion of Amonafide and metabolites. –●–, Amonafide; –▲–, *N*-oxide metabolite; –■–, *N*-demethylated metabolite. Data are presented as mean ± SD from six dogs

creted in the bile in 6 h (Fig. 3) as the unchanged drug. Amonafide penetrates into the CSF as early as 5 min after the end of injection (Fig. 1). Its peak concentration appears to occur 20–25 min after injection, and the average CSF level was about 3% of the concurrent plasma level.

Distribution

At autopsy, carried out 6 h after dosing, the liver had accumulated the highest percentage of the administered dose as the unchanged drug (0.23%), followed by the stomach (0.11%), lung (0.045%), kidney (0.041%), and pancreas (0.031%). The heart, small and large intestines, brain, spleen, prostate, muscle, and lymph nodes each retained less than 0.02% of the original dose (Fig. 4). Expressed in ng/g wet tissue, the Amonafide distribution assumed a different pattern. Six hours after drug administration, the kidney, pancreas, lymph node, liver, stomach, and testes were found to have Amonafide concentrations of 0.5 μg/g or more (Fig. 5). Drug concentrations in the prostate, urinary bladder, gall bladder, small intestine, heart, muscle, large intestine, brain, and spleen were between 0.5 and 0.04 μg/ml. After 24 h, however, no detectable amount of Amonafide was observed in any of the tissues.

Metabolism

Amonafide was metabolized extensively to three major and one or two minor metabolites. Figure 6 shows the total ion-current trace obtained by LC/MS analysis of HPLC-separated parent drug and metabolites. Two distinct chromatographic peaks were detected with retention times of 5.1 and 6.5 min, respectively. The mass spectrum of the major metabolite (retention time, 5.1 min) (Fig. 7) was found to be identical to that of an authentic, synthetically derived, *N*-oxide standard of Amonafide, and was distinctly different from the mass spectral data obtained for the parent drug (Fig. 8). This metabolite was characterized by a base peak at M/c 270 and prominent ions at M/Z 239, 284, and 300. The same ions are also present in the mass spectrum of the parent drug. If we assume that M/Z 300 represents the protonated molecular ion for the major

Table 1. Pharmacokinetic parameters of Amonafide and *N*-oxide Amonafide

	Plasma $t_{1/2}$			Vd	Clearance	Excretion (%)[a]	
	α, min	β, min	γ, h	l/kg	l/kg/h	Urine, 24 h	Bile, 6 h
Amonafide	2.4±0.4[b]	26.8±3.7	21.7±4.0	12.8±0.5	0.56±0.16	9.5±0.2	7.4±1.4
	α, min	β, h					
N-oxide Amonafide	24.8	28.6		12.1	0.3	11.4	16.7

[a] Data are expressed as a percentage of administered dose
[b] Data are presented as mean ± SD of data from 5 dogs

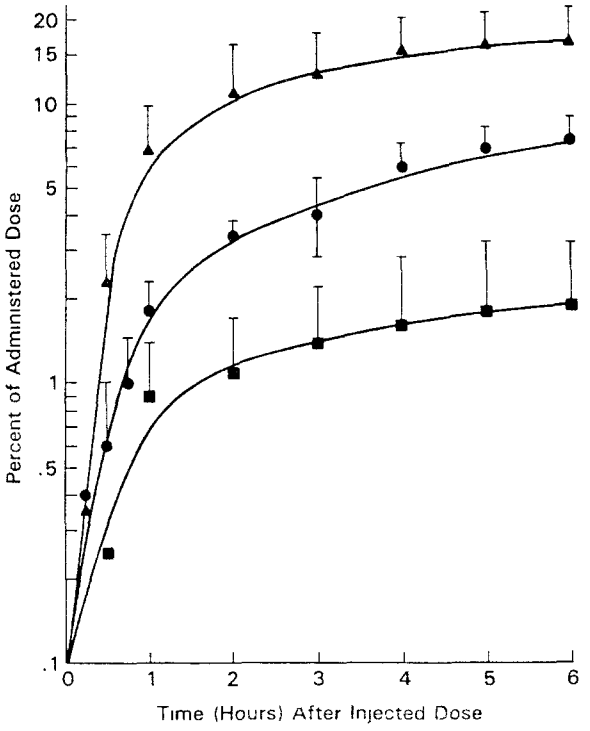

Fig. 3. Cumulative biliary excretion of Amonafide and metabolites. –●–, Amonafide; –▲–, *N*-oxide metabolite; –■–, *N*-demethylated metabolite. Data are presented as mean ± SD from six dogs

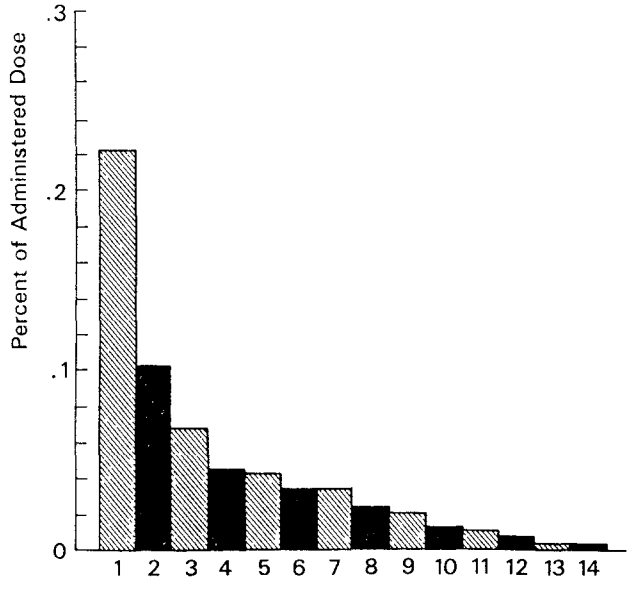

Fig. 4. Tissue distribution of Amonafide (percentage of administered dose). Data are reported as the mean of analyses from three dogs. Liver (1), stomach (2), small intestine (3), kidney (4), lung (5), pancreas (6), gall bladder (7), testes (8), heart (9), large intestine (10), prostate (11), brain (12), spleen (13), urinary bladder (14)

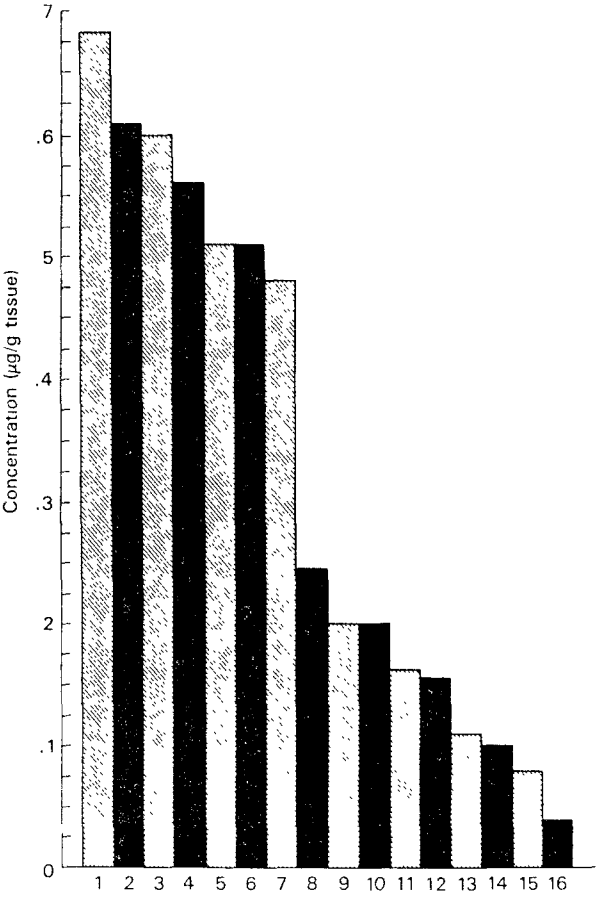

Fig. 5. Tissue concentration of Amonafide. Data are presented as the mean of analyses from three dogs. *1*, kidney; *2*, pancreas; *3*, lymph nodes; *4*, liver; *5*, stomach; *6*, testis; *7*, prostate; *8*, urinary bladder; *9*, gall bladder; *10*, lung; *11*, small intestine; *12*, heart; *13*, muscle; *14*, large intestine; *15*, brain; *16*, spleen

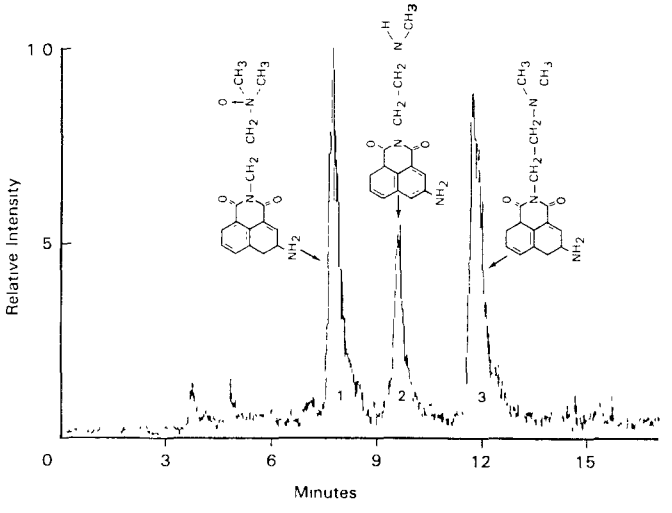

Fig. 6. Total LC/MS trace of Amonafide and the *N*-oxide and *N*-demethylated metabolites

metabolite, the metabolite must be arising by oxidation of the parent compound, since the difference in molecular weight is 16 atomic mass units (a. m. u.). It is also important to note that three common fragment ions are more intense for the metabolite than Amonafide. This suggests that oxidation, presumably enzymatic, has probably taken place in the side chain rather than the naphthalimide ring. Since there are four possible sites of metabolic oxidation in the side chain, three aliphatic carbonates, as well as the *N*-dimethylamino nitrogen atom, the mass spectrum alone

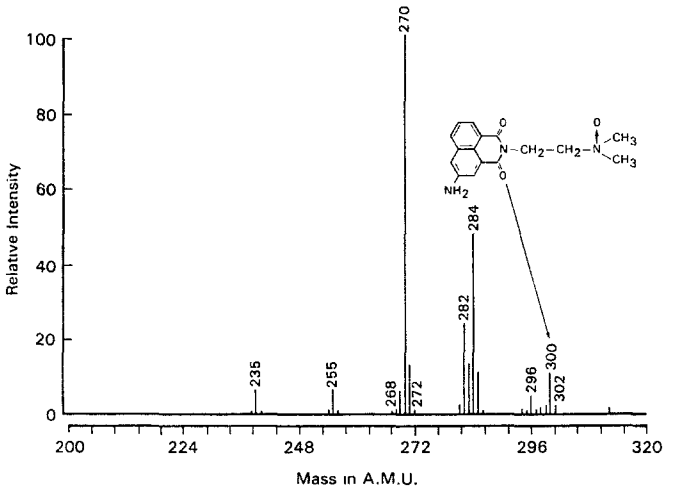

Fig. 7. Mass spectrometrice analysis of the *N*-oxide metabolite of Amonafide. Data were obtained from analysis of a urinary sample subjected to thin-layer chromatography and then to HPLC-thermospray-MS analysis as described in Materials and methods

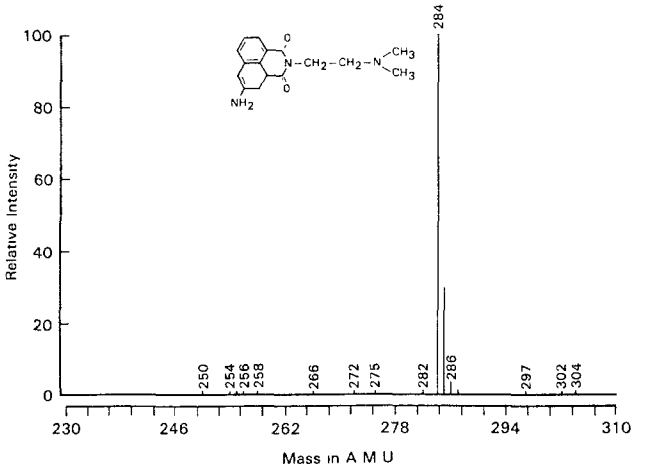

Fig. 8. Mass spectrometric analysis of Amonafide. Data were obtained as described in Fig. 7

cannot be used to determine unequivocally the actual site of oxidation. It is known, however, that dimethyl-substituted *N*-oxide can undergo thermal demethylation by loss of formaldehyde, and that the mass spectrum of the metabolite has an intense ion at M/Z 270, which would correspond to this chemical process. Thus, the mass spectral data in combination with the retention time strongly suggest that this major metabolite is the *N*-oxide of Amonafide.

In dog plasma, only the *N*-oxide of Amonafide was present. However, in the urine and bile, there were two other major metabolites besides the *N*-oxide. These were identified as *N*-demethylated and hydroxylated products of Amonafide. The *N*-demethylated metabolite has ions at M/e 270 (base peak) and 239 (Fig. 9). The hydroxylated metabolite has graduated molecular ions at M/e 300.

The *N*-oxide metabolite of Amonafide present in the plasma peaked at 20 min after the end of infusion (Fig. 1), and then cleared biphasically from the plasma, with an initial $t_{1/2}$ of 24.8 min and a terminal $t_{1/2}$ of 28.6 h. Its clearance rate was 0.3 l/kg/h, and its apparent volume of distribution was 12.1 l/kg. The cumulative excretion was

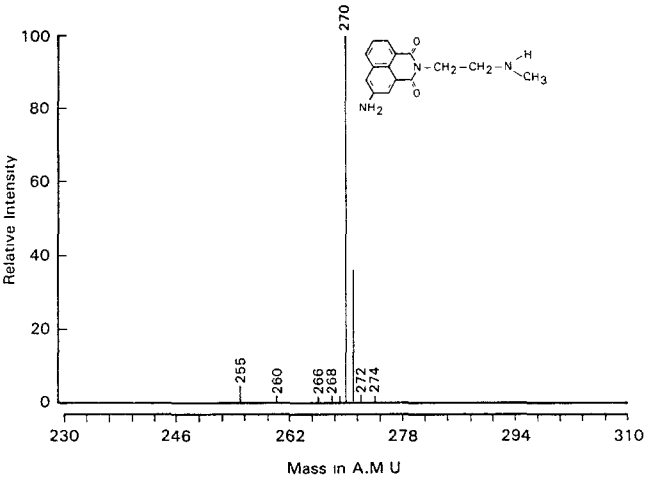

Fig. 9. Mass spectrometric analysis of the *N*-demethylated derivative of Amonafide. Data were obtained as described in Fig. 7

11.4%±8.6% in 24 h in the urine and 16.7%±5.4% in 6 h in the bile (Table 1).

The cumulative excretion of the *N*-demethylated metabolite in 24 h was 3.8%±1.8% in the urine (Fig. 2) and 4.8%±8% in the bile (Fig. 3). A total of 5.3%±1.4% of the hydroxylated Amonafide was excreted in the urine in 24 h, and 1.9%±1.1% appeared in the bile.

The present study is an excellent demonstration of the species-to-species variations in drug metabolism. Since dogs do not possess a significant ability to conjugate via the *N*-acetylation pathway for aromatic amines such as the primary amino group in Amonafide, the *N*-acetylated metabolite is not the major metabolite in dogs. In contrast, Amonafide is extensively metabolized in humans via *N*-acetylation [4]. Also, because this drug's principal means of metabolism is via *N*-acetylation in man, the importance of variations in acetylation rates between individuals may also be important. Since dogs are unable to acetylate amino groups attached to the aromatic ring, this may at least in part account for the difference in toxicity seen in the two different species.

References

1. Andersson BS, Beran M, Bakic M, Silberman LE, Newman RA, Zwelling LA (1987) In vitro toxicity and DNA cleaving capacity of benzisoquinolinedione (Nafidimide NSC 308847) in human leukemia. Canc Res 47: 1040–1044
2. Brana MF, Castellano JM, Roldan CM, Santos A, Vazquez D, Jimenez A (1980) Synthesis and mode(s) of action of new series of imide derivatives of 3-nitro-1,8 naphthalic acid. Cancer Chemother Pharmacol 4: 61–66
3. Brana MF, Sanz MA, Castellano JM, Roldan MC, Roldan C (1981) Synthesis and cytostatic activity of benz[de]isoquinoline-1,3-dione's structure-activity relationships. Eur J Med Chem 16: 207–212
4. Felder TB, McLean MA, Vestal ML, Lu K, Farquhar D, Legha SS, Shah R, Newman RA. Pharmacokinetics and metabolism of the antitumor drug amonafide (NSC 308847) in humans. Drug Metab and Dispos (in press)
5. Lu K, Savaraj N, Newman RA (1985) Liquid chromatographic separation and quantitation of benzisoquinolinedione (NSC-308847 Nafidimide) from biological fluids. J Chromatogr 345: 408–412

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