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Disposition and metabolism of the antitumor agent pyrazine-2-diazohydroxide in mouse and beagle dog*

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Summary. The pharmacokinetics and metabolism of pyrazine-2-diazohydroxide have been studied in the beagle dog and mouse. When pyrazine-2-diazohydroxide was administered to beagle dogs at a dose of 18.6 mg/kg (428 mg/m^2) by i.v. bolus, the plasma half-life ($t_{1/2}$) was 7.3 min, the apparent volume of distribution (V_d) 577 ml/kg, and the total body clearance (Cl) 55 ml/min per kg. In mice given pyrazine-2-diazohydroxide by i.v. bolus at 100 mg/kg (428 mg/m^2), the $t_{1/2}$ was 5.8 min, the V_d 250 ml/kg, and the Cl 30 ml/min per kg. When [2-^{14}C]pyrazine-2-diazohydroxide was infused i.v. to mice at 100 mg/kg over 8 h, the Cl for parent drug was 122 ml/min per kg. The major product formed from pyrazine-2-diazohydroxide was 2-hydroxypyrazine, which accounted for 80% of the total radioactivity in the plasma after a 6-h drug infusion. There were three other metabolites in plasma, two more polar than pyrazine-2-diazohydroxide, which accounted for 7% of the radioactivity, and one less polar, which accounted for 5% of the radioactivity. Following an i.v. bolus dose of [2-^{14}C]pyrazine-2-diazohydroxide, 79% of the radioactivity was excreted in the urine in 24 h, 3% in the feces, and 0.4% in the expired air; 18% remained in the carcass. The liver and kidney showed the highest tissue levels of radioactivity. 2-Hydroxypyrazine accounted for 45% of the urinary radioactivity, pyrazine-2-diazohydroxide for 14%, and a glucuronide or sulfate conjugate of 2-hydroxypyrazine for 17%. Twenty-four percent of the radioactivity eluted near the void volume on high-performance liquid chromatography and was not identified.

Introduction

Pyrazine-2-diazohydroxide exhibits antitumor activity when administered i.p. in a number of animal tumor models including murine L1210 and P388 leukemias, B-16 melanoma, M5076 sarcoma, and in a human MX-1 mammary xenograft [4]. The structure of pyrazine-2-diazohydroxide is shown in Fig. 1. Pyrazine-2-diazohydroxide is currently being considered by the National Cancer Institute, USA for clinical trial. Previous work has shown pyrazine-2-diazohydroxide to be broken down rapidly under mildly acidic conditions but to be more stable under alkaline conditions [3, 6]. We now report the metabolism and disposition of pyrazine-2-diazohydroxide in the mouse and beagle dogs.

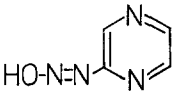

Fig. 1. Structure of pyrazine-2-diazohydroxide

Materials and methods

Drugs. Pyrazine-2-diazohydroxide (NSC 361456) and [2-^{14}C]pyrazine-2-diazohydroxide (specific activity 65.1 μCi/mg) were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md, USA. The purity of the [2-^{14}C]pyrazine-2-diazohydroxide was determined by high-performance liquid chromatography (HPLC) to be 98%. Pyrazine-2-diazohydroxide was formulated immediately prior to administration as a 20-mg/ml solution in 0.9% sodium chloride adjusted to pH 10.0 with 1 *N* sodium hydroxide (pH 10 saline). 2-Hydroxypyrazine was prepared by acidification of a 100 μg/ml solution of pyrazine-2-diazohydroxide in water to pH 4.8 with 1 *N* HCl. The solution was freeze-dried, and the purity of the product was confirmed by HPLC analysis. The NMR spectrum (acetamide) of the product showed hydrogen shifts consistent with it being a hydroxypyrazine. Signals were observed at γ 8.8 (d, 1H, J = 2 Hz), γ 8.4 (d, 1H, J = 4 Hz), and γ 8.6 (dd, 1H, J = 4, 2 Hz). The structure of the compound was also confirmed by IR spectroscopy. The spectra showed strong peaks at 1595, 1640, and 1680 cm^{-1} which are consistent with C-H bonds of an aromatic compound. At 3400 and 2600 cm^{-1} there were broad peaks consistent with OH stretch. Since there was no peak observed between 1700 and 1800 cm^{-1}, this suggests that the compound exists predominantly as the hydroxyl rather than the keto form.

Assays. Nonlabeled pyrazine-2-diazohydroxide was assayed by a gas chromatographic (GC) procedure previously described [6]. Briefly, 0.1–0.5 ml plasma was mixed with 250 μl concentrated hydrochloric acid, followed, after thorough mixing, by 375 μl 10 *N* sodium hydroxide. The mixture was shaken for 20 min with 1 ml ethyl acetate containing 0.5 μg 2,5-dimethylpyrazine as an internal standard and then centrifuged for 10 min at 1000 *g*. The upper

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organic layer was removed and 1.5 µl taken for GC analysis. A standard curve of pyrazine-2-diazohydroxide in fresh plasma was prepared in the same way. To correct for any 2-chloropyrazine originally present, a 0.5-ml control sample of plasma was treated in the same way, except that the concentrated hydrochloric acid and 10 *N* sodium hydroxide were mixed before adding the plasma containing the pyrazine-2-diazohydroxide. The lower limit of sensitivity of the assay for pyrazine-2-diazohydroxide was 50 ng/ml.

An HPLC procedure was developed for the measurement of [2-^{14}C]pyrazine-2-diazohydroxide and its metabolites. Proteins were precipitated with an equal volume of methanol, pH 9, at 4° C for 1 h and the mixture centrifuged for 2 min at 11000 *g*. A 50-µl aliquot of the supernatant solution was taken for HPLC analysis. The HPLC system employed a 25-cm Hibar II RP-18 5 µ*M* column (Merck, Darmstadt, FRG) and isocratic separation with a solvent of 50 m*M* tetrabutyl ammonium phosphate, pH 8.0, (Millipore Corp., Milford, Mass) containing 5% methanol at a flow rate of 1.0 ml/min. Eluting compounds were detected by their absorbance at 300 nm on a Hewlett-Packard 798575A variable wavelength detector. Radiolabeled pyrazine-2-diazohydroxide and its metabolites were detected using a Raytest-Ramona radiochromatographic flow detector (IN/US Instruments, Fairfield, NJ). The output from both detectors was fed into a Hewlett-Packard 79850B liquid chromatograph terminal and peak areas integrated.

Pharmacokinetic studies. Pyrazine-2-diazohydroxide at a dose of 100 mg/kg (428 mg/m^2) was administered by rapid i.v. injection (< 30 s) into the tail vein of male CDF_1 mice weighing 23–25 g held in a Broome-type restraint. Blood was collected from groups of three mice at 0, 2, 4, 6, 8, 10, 15, 20, 30, and 60 min. The mice were lightly anesthetized with diethyl ether, and as soon as rapid limb movements had ceased the animals were exsanguinated by bleeding from the retro-orbital venous plexus [2] into chilled heparinized 1.5-ml centrifuge tubes. The times represent the midpoints of the blood collection period which took approximately 30 s. The blood was immediately centrifuged at 10000 *g* for 2 min in a microcentrifuge and 0.1 ml plasma taken for GC analysis of pyrazine-2-diazohydroxide.

Pyrazine-2-diazohydroxide was administered to female beagle dogs weighing 10–14.5 kg by i.v. bolus injection over 1 min at a dose of 18.6 mg/kg (428 mg/m^2). Dogs were placed in a Pavlov-type sling and the drug was injected through a Teflon catheter (Angiocath 18 gauge, Deseret Co., Sandy, Utah, USA) into a cephalic vein. Blood samples of 3 ml were drawn into a syringe from a second Teflon catheter in the other cephalic vein at 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, and 90 min following drug administration. The blood was immediately transferred to chilled heparinized tubes, which were centrifuged at 10000 *g* for 2 min to separate plasma. Duplicate samples of 0.5 ml plasma were taken for GC assay of pyrazine-2-diazohydroxide.

Plasma drug concentration data were subjected to nonlinear least-squares regression analysis using the NONLIN pharmacokinetic computer program [1] with a weighting factor of $1/y^2$. Pharmacokinetic parameters were calculated according to Wagner [5].

Studies using [2-^{14}C]pyrazine-2-diazohydroxide. Male CDF_1 mice weighing 20–23 g were infused through an indwelling PE-10 polyethylene catheter (Intramedic, Clay Adams) in the tail vein with [2-^{14}C]pyrazine-2-diazohydroxide diluted with unlabeled pyrazine-2-diazohydroxide to 1.09 µCi/mg at a rate of 208 µg/min/kg for 8 h or a total dose of 100 mg/kg. The formulated drug was infused at a rate of 5 µl/min using a constant infusion pump (Harvard Apparatus, Natick, Mass, USA). Groups of three mice were exsanguinated at 1, 2, 4, 6, and 8 h, plasma separated from the blood as before and assayed for pyrazine-2-diazohydroxide by GC analysis. Total radioactivity in plasma was measured in 50 µl aliquots by liquid scintillation counting.

In a second experiment, a group of six male CDF_1 mice received [2-^{14}C]pyrazine-2-diazohydroxide by infusion as before but were killed after 6 h. The plasma was assayed for radioactive drug and metabolites by HPLC. Radioactivity in the tissues was measured by preparing a 5% w/v homogenate of tissues in distilled water. To a 1-ml aliquot of the homogenate was added 2.8 ml of tissue solubilizer (Beckman Instruments, Fullerton, Calif.) and the mixture was allowed to stand at room temperature overnight. The mixture was decolorized with 0.25 ml 30% H_2O_2 at room temperature overnight followed by addition of 0.25 ml 1 *N* HCl. Ten milliliters of Insta gel liquid scintillant (Packard Instrument, Downers Grove, Ill) was added, and radioactivity was determined by liquid scintillation counting.

In a third experiment a group of five male CDF_1 mice received [2-^{14}C]pyrazine-2-diazohydroxide diluted with unlabeled drug to 0.5 µCi/mg at a total dose of 100 mg/kg, by rapid i.v. injection into the tail vein. The animals were placed in an all-glass metabolism chamber (Bellacour Glass Company, Laurelton, NY). Urine and feces were collected on dry ice for 24 h, and $^{14}CO_2$ in the expired air was collected in 10% KOH. Following completion of the experiment the mice were killed and the carcasses dissolved in aqua regia for 7 days. Radioactivity in the urine, feces, expired air, and digested carcasses was determined by liquid scintillation counting. Radioactive drug and metabolites in 50 µl urine were determined by HPLC. A urine sample of 2 ml was mixed with 0.2 ml 0.1 *M* sodium acetate, pH 6.4, containing 2000 U β-glucuronidase and 426 U sulfatase (Sigma Chemical Co., St Louis, Mo, USA) and incubated at 37° C for 24 h. A sample of the urine incubated with sodium acetate alone was included as a reference blank. Following treatment the urine was analyzed by HPLC for radioactive peaks.

Plasma protein binding of [2-^{14}C]pyrazine-2-diazohydroxide. The precipitated protein pellet from plasma of mice that had received [2-^{14}C]pyrazine-2-diazohydroxide by 6-h infusion was washed three times by resuspending in 2 ml methanol. The pellet was then solubilized and the radioactivity determined by liquid scintillation counting as previously described.

Results

Pharmacokinetic studies

Plasma concentrations of pyrazine-2-diazohydroxide in male CDF_1 mice given an i. v. bolus dose of pyrazine-2-diazohydroxide of 100 mg/kg (428 mg/m^2) are shown in Fig. 2. The elimination of pyrazine-2-diazohydroxide was rapid and followed a monoexponential decay. The half-

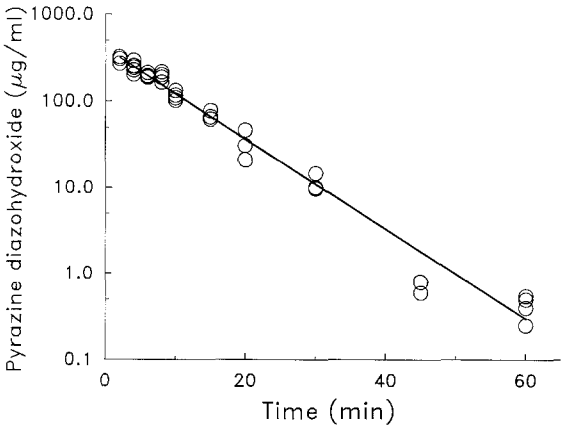

Fig. 2. Plasma pyrazine-2-diazohydroxide in male CDF_1 mice after i.v. bolus administration of pyrazine-2-diazohydroxide, 100 mg/kg. Each *circle* represents one animal. The *continuous line* is the computer-generated fit to the data

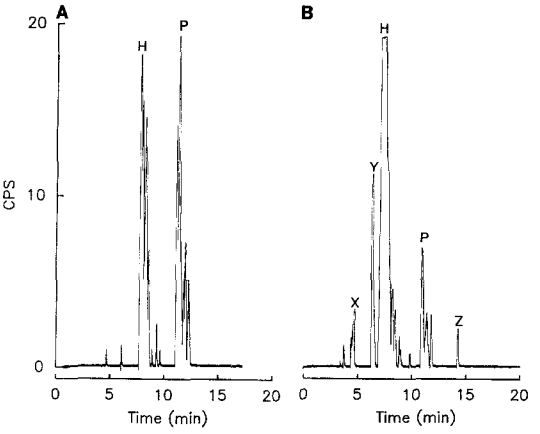

Fig. 5. HPLC radiochromatograms of **A** [2-^{14}C]pyrazine-2-diazohydroxide following partial hydrolysis in pH 7.0 buffer and **B** plasma of mice administered [2-^{14}C]pyrazine-2-diazohydroxide, 75 mg/kg over 6 h. The *peaks* are *P*, pyrazine-2-diazohydroxide and *H*, 2-hydroxypyrazine, identified by comparison to reference compounds. *X*, *Y*, and *Z* are unidentified metabolite peaks

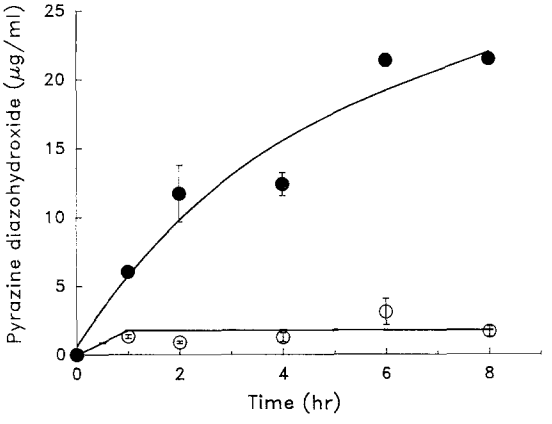

Fig. 3. Plasma pyrazine-2-diazohydroxide and total radioactivity following i.v. infusion of [2-^{14}C]pyrazine-2-diazohydroxide to male CDF_1 mice at 100 mg/kg over 8 h. Each *point* is the mean of from animals. *Bars* are SE of mean. ○, Pyrazine-2-diazohydroxide determined by gc assay; ●, total radioactivity expressed in [2-^{14}C]pyrazine-2-diazohydroxide equivalents

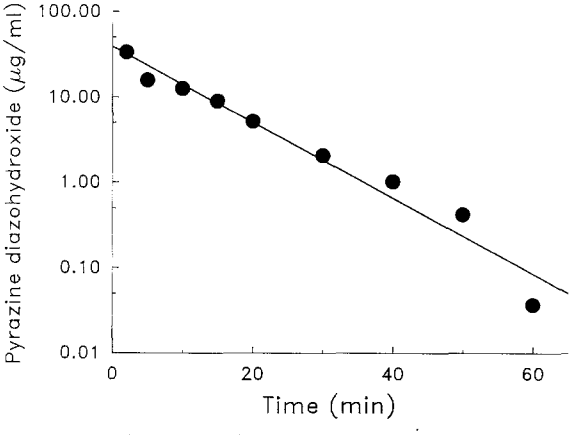

Fig. 4. Pyrazine-2-diazohydroxide plasma elimination in a female beagle dog after i.v. injection of pyrazine-2-diazohydroxide 18.6 mg/kg (428.6 mg/m^2). The *continuous line* is the computer-generated fit to the data

life of pyrazine-2-diazohydroxide in plasma (± SE) was 5.8 ± 0.3 min, the apparent volume of distribution 250.3 ± 11.2 ml/kg, and the total body clearance 29.8 ± 0.9 ml/min per kg. Infusion of [2-^{14}C]pyrazine-2-diazohydroxide to mice for 8 h resulted in a steady-state plasma level of unchanged pyrazine-2-diazohydroxide of 1.7 μg/ml within 1 h of infusion (Fig. 3). This gave a total body clearance of 122 ml/min per kg. Plasma total radioactivity continued to rise throughout the infusion period.

Plasma concentrations of pyrazine-2-diazohydroxide in a beagle dog given an i.v. bolus dose of pyrazine-2-diazohydroxide of 18.6 mg/kg (428 mg/m^2) are shown in Fig. 4. The plasma elimination of pyrazine-2-diazohydroxide was monoexponential with a mean half-life in three dogs (± SE) of 7.3 ± 0.7 min, an apparent volume of distribution of 576.6 ± 46.0 ml/kg and a total body clearance 55.0 ± 1.2 ml/min per kg.

Metabolism studies

The reversed-phase HPLC assay procedure gave good separation of [2-^{14}C]pyrazine-2-diazohydroxide and its breakdown products and metabolites (Fig. 5). Plasma from mice administered [2-^{14}C]pyrazine-2-diazohydroxide for 6 h by continuous i.v. infusion showed several peaks in addition to a small amount of parent drug (Fig. 5). Parent drug accounted for 7% of the total radioactivity (Fig. 5). The major peak in plasma was 2-hydroxypyrazine, which was identified by its retention time compared with the reference compound and accounted for 80% of the total radioactivity. Two minor peaks more polar than pyrazine-2-diazohydroxide accounted for 7% of the radioactivity, and a peak less polar than pyrazine-2-diazohydroxide for 5% of the radioactivity. There was 1.0% radioactivity remaining with the washed precipitated plasma protein fraction, indicating a small amount of covalent binding of pyrazine-2-diazohydroxide to plasma proteins. The distribution of radioactivity in the tissues of mice infused with [2-^{14}C]pyrazine-2-diazohydroxide is shown in Table 1. The highest concentration of radioactivity was found in the liver, followed by the kidney.

Table 1. Tissue distribution of radioactivity following i.v. administration of [2-^{14}C]pyrazine-2-diazohydroxide to mice

	Tissue concentration nCi/g or ml
Blood	8.4
Plasma	12.1
Liver	45.0
Kidney	28.0
Brain	7.2
Heart	4.9
Lung	6.8

Mice each received [2-^{14}C]pyrazine-2-diazohydroxide, 75 mg/kg, 82 μCi/kg, by i.v. infusion over 6 h. The tissues were homogenized and total radioactivity determined. Values are the means ± SE of mean from 3 mice in each case

Recovery of radioactivity from a group of mice 24 h after administration of an i.v. bolus dose of [2-^{14}C]pyrazine-2-diazohydroxide was 78.6% in the urine, 3.3% in the feces, 0.4% in the expired air, and 17.5% in the carcass, with a total recovery of 99.8%. A radiochromatogram of the urine of the mice administered [2-^{14}C]pyrazine-2-diazohydroxide is shown in Fig. 6. Unchanged pyrazine-2-diazohydroxide accounted for 13.6% of the total radioactivity in the urine over 24 h. The major radioactive peak in urine was 2-hydroxypyrazine which accounted for 44.9% of total radioactivity, with a smaller more polar metabolite peak accounting for 17.1% of the total activity, and 24.4% of the activity eluting near the void volume. Incubation of the urine with β-glucuronidase and sulfatase for 24 h resulted in the disappearance of the small more polar metabolite peak and an increase in the size of the 2-hydroxypyrazine peak. Control incubations without enzymes for 24 h showed that the metabolites were stable over this time, although the small amount of pyrazine-2-diazohydroxide disappeared during the incubation (Fig. 6). The results suggests that the more polar metabolite is a glucuronide or sulfate conjugate of 2-hydroxypyrazine. The amount of radioactivity eluting near the void volume was not altered by β-glucuronidase and sulfatase treatment.

Discussion

Pyrazine-2-diazohydroxide exhibits rapid monophasic elimination from the plasma of mouse and dog with half-lives of 5.8 and 7.3 min, respectively. Previous studies have shown that pyrazine-2-diazohydroxide added to mouse plasma in vitro is rapidly broken down with a half-life of 14.4 min at 37° C [6]. It is likely, therefore, that the rapid disappearance of pyrazine-2-diazohydroxide from plasma in vivo is due to the same process of chemical breakdown. The main product of the breakdown of pyrazine-2-diazohydroxide in aqueous media is 2-hydroxypyrazine formed by the nucleophilic attack of water [3]. 2-Hydroxypyrazine was also found to be the major product formed from pyrazine-2-diazohydroxide in vivo. 2-Hydroxypyrazine, unlike pyrazine-2-diazohydroxide, exhibits no cytotoxicity against tumor cells in culture (D. J. Moore, Melder, and G. Powis, unpublished observations). After 6-h infusion of radiolabeled pyrazine-2-diazohydroxide to mice, pyrazine-2-diazohydroxide accounted for 7% of the total radioactivity in plasma and 2-hydroxypyrazine for 80%. There were three unidentified metabolite peaks in plasma, two more polar and one less polar than pyrazine-2-diazohydroxide, which together accounted for 12% of the total radioactivity. A small amount of radioactivity remained bound to plasma protein after precipitation and washing, indicating that pyrazine-2-diazohydroxide was reacting covalently with a component in the plasma protein.

The majority of the pyrazine-2-diazohydroxide administered to mice was excreted in the urine in 24 h. After administration of radiolabeled pyrazine-2-diazohydroxide, 78.6% of the radioactivity was found in the urine, 3.3% in the feces, which suggests little or no biliary excretion, 17.5% remained in the carcass, and 0.4% of radioactivity

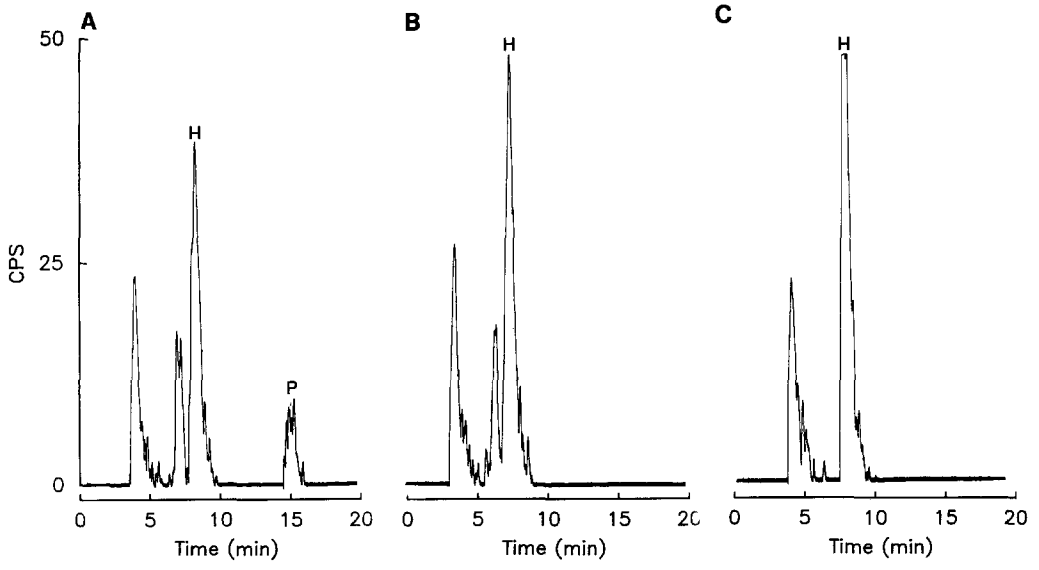

Fig. 6. HPLC radiochromatograms of 24-h urine from mice administered [2-^{14}C]pyrazine-2-diazohydroxide, 100 mg/kg by i.v. bolus. **A** Untreated urine; **B** urine treated with 0.1 *M* sodium acetate, pH 6.4, for 24 h; **C** urine treated with β-glucuronidase and sulfatase in 0.1 *M* sodium acetate, pH 6.4, for 24 h. The *peaks* are *P* pyrazine-2-diazohydroxide and *H* 2-hydroxypyrazine, identified by comparison to reference compounds

was found in the expired air. The lack of radioactive CO_2 in the breath shows that the pyrazine ring is not cleaved by metabolism. Tissue-distribution studies showed that liver and kidney had the highest levels of pyrazine-2-diazohydroxide-derived radioactivity. Studies of the radioactivity excreted in mouse urine over 24 h showed that pyrazine-2-diazohydroxide accounted for 14% of the radioactivity and 2-hydroxypyrazine for 45%. A peak which eluted near the void volume accounted for 24% of the radioactivity while a metabolite more polar than 2-hydroxypyrazine accounted for 17%. Treatment of the urine with β-glucuronidase and sulfatase resulted in the disappearance of the smaller peak suggesting it to be a conjugate. It was not possible to unequivocally identify the conjugate but the concomitant growth of the 2-hydroxypyrazine peak suggests that it was a sulfate or a glucuronide conjugate of 2-hydroxypyrazine. The radioactivity eluting near the void volume did not appear to be effected by β-glucuronidase or sulfatase treatment and may represent degradation products resulting from aklylation of biological molecules by pyrazine-2-diazohydroxide. The less polar pyrazine-2-diazohydroxide metabolite seen in plasma was not detected in urine.

Pyrazine-2-diazohydroxide exhibited a relatively similar total body clearance following its i.v. bolus administration in mouse (30 ml/min per kg) and dog (55 ml/min per kg). Infusion of the drug to mice over 8 h gave a much larger total body clearance of 122 ml/min per kg. The reason for the apparent dose-dependent elimination of pyrazine-2-diazohydroxide, when most elimination appears to be by chemical degradation, is not known. It might represent depletion of a pool of endogenous compounds with which pyrazine-2-diazohydroxide reacts following bolus administration of the drug. When pyrazine-2-diazohydroxide is infused there could be time for repletion of this pool of endogenous compounds and more rapid elimination of the drug. Pyrazine-2-diazohydroxide does not react directly with glutathione (J. I. Brodfuehrer and G. Powis, unpublished observations), which would rule out cellular glutathione as the site of reaction. Other cellular thiols or other nucleophiles could, however, be involved.

In summary, the pharmacokinetics and metabolism of pyrazine-2-diazohydroxide have been studied in the mouse and beagle dog. The compound is rapidly eliminated in both species. The major product formed from pyrazine-2-diazohydroxide is the non-cytotoxic 2-hydroxypyrazine by spontaneous degradation. Metabolites of pyrazine-2-diazohydroxide, in addition to 2-hydroxypyrazine, have been found in the plasma and the urine of the mouse. One of these metabolites in urine is a glucuronide or sulfate conjugate of 2-hydroxypyrazine. The major route for elimination of pyrazine-2-diazohydroxide and its products is in the urine.

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