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Preclinical pharmacologic studies of the new antitumor agent carmethizole (NSC-602668) in the mouse and beagle dog

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Summary. The chemical breakdown of carmethizole [1-methyl-2-methylthio-4,5-bis-(hydroxymethyl)imidazole-4′,5′-bis(*N*-methylcarbamate)hydrochloride] and its pharmacokinetics in the mouse and beagle dog were studied. Carmethizole was relatively unstable in aqueous media, having a half-life of ≤ 1 h in 0.9% sodium chloride, human whole blood, human plasma, and dog urine at 37° C. Its major breakdown product in 0.9% sodium chloride and pH 5.0 sodium phosphate buffer was carmethizole diol. When carmethizole was added to pH 7.0 or pH 9.0 sodium phosphate buffer, the major breakdown product was carmethizole diol-4′-monophosphate. Carmethizole reacted directly with glutathione at pH 8.0, forming a glutathione adduct of carmethizole monocarbamate. Elimination of the drug from the plasma of the beagle dog following i.v. bolus doses of 22.4 and 4.3 mg/kg was biphasic. At these doses the terminal half-life was 39 and 46 min, respectively, and the respective total body clearance was 4.6 and 7.7 ml/min per kg. The 22.4 mg/kg dose was lethal to the beagle dog by day 4. Elimination of carmethizole from the plasma of mice following an i.v. bolus dose of 115 mg/kg was monoexponential, with a half-life of 11.6 min and a total body plasma clearance of 43.6 ml/min per kg. When the drug was infused at 230 mg/kg over 8 h into mice, the total body clearance was 40.8 ml/min per kg. Following the i.v. bolus administration of carmethizole to mice, 30% of the total dose was excreted in urine over 3 h as carmethizole diol, 10%, as carmethizole diol-sulfate, 3.4%, as carmethizole 4′-monocarbamate, and 2.4%, as unchanged drug.

Introduction

Carmethizole [1-methyl-2-methylthio-4,5-bis(hydroxymethyl)imidazole-4′,5′-bis(*N*-methylcarbamate)hydrochloride] was synthesized as a rationally designed congener of the experimental antitumor agent pyrrolizidine biscarbamate in an attempt to develop a compound with greater solubility and improved stability [1, 7]. The structure of carmethizole is shown in Fig. 1. This drug has exhibited antitumor activity when given i.p. in a number of animal tumor models including P388 leukemia, i.p. implanted LOX melanoma, and the subrenal capsule-implanted mammary human xenograft [1, 7]. Carmethizole is currently being developed by the National Cancer Institute (USA) for clinical trial. This study reports the pH and temperature stability of carmethizole in salt solutions as a guide to its formulation, stability in biological fluids, and reactivity with the cellular nucleophile glutathione and describes pharmacokinetic studies in the mouse and beagle dog.

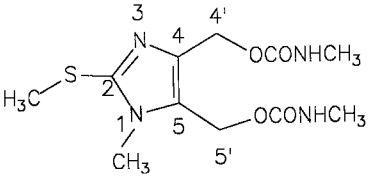

Fig. 1. Structure of carmethizole

Methods and materials

Drugs. Carmethizole (NSC-602668) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md, USA). Its purity, determined by high-performance liquid chromatography (HPLC), was >99%. Carmethizole 4′-monocarbamate [1-methyl-2-methylthio-5-(hydroxymethyl)imidazole-4-(*N*-methylcarbamate)hydrochloride] and carmethizole diol [1-methyl-2-methylthio-4,5-bis(hydroxymethyl)imidazole hydrochloride] were kindly supplied by Dr. Wayne K. Anderson, Department of Medicinal Chemistry, State University of New York (Buffalo, NY). Carmethizole diol-4′-monophosphate was prepared by heating a 10 mg/ml solution of carmethizole in 0.1 *M* sodium phosphate buffer (pH 8.0) at 37° C for 24 h. The phosphate adduct was separated from carmethizole and other products using a 3-ml C-18 extraction column (J. T. Baker, Phillipsburg, NJ) and eluting with 2 ml water. Under these conditions carmethizole diol and unreacted carmethizole remained bound to the column, whereas carmethizole diol-4′-monophosphate eluted with the water. The glutathione adduct of carmethizole was prepared by heating a solution of 10 mg/ml carmethizole and 100 mg/ml glutathione (reduced form) in water (adjusted to pH 8.0 with 5 *N* NaOH) at 37° C for 3 h. The glutathione adduct was separated from carmethizole and the other breakdown products using a 3-ml C-18 extraction column preequilibrated with 3 m*M* sodium phosphate buffer (pH 7.2) and

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eluted with water. Unreacted glutathione eluted with the first 2 ml water and the glutathione adduct of carmethizole, with 5–8 ml water. Carmethizole diol and unreacted carmethizole remained bound to the column. 4-Hydroxybiphenyl was purchased from Aldrich Chemical Company (Milwaukee, Wis, USA). β-Glucuronidase, aryl sulfatase and glutathione (reduced form) were purchased from Sigma Chemical Co. (St. Louis, Mo). All other chemicals were reagent grade.

Preparation of samples. A 0.5 ml sample of buffer, urine, plasma, or heparinized blood containing carmethizole was mixed with 2 ml ethyl acetate and 1 μg 4-hydroxybiphenyl as an internal standard. The mixture was shaken for 15 min on a mechanical shaker and then centrifuged for 10 min at 3,000 *g*. The organic layer was withdrawn and taken to dryness under N_2. The residue could be stored frozen at this stage or dissolved in 200 μl methanol. A 100-μl aliquot of the methanol solution was taken for HPLC analysis. Preliminary studies showed that carmethizole was stable in methanol at room temperature with <7% degradation over 48 h. Since the breakdown products of carmethizole extracted poorly into ethyl acetate, a modified procedure without organic solvent extraction was used for their measurement in buffer or urine. A 50-μl aliquot of the sample was taken for direct injection onto the HPLC. Urine was diluted 1:10 with 0.9% sodium chloride prior to injection. Since this procedure lacked the sensitivity of the ethyl acetate extraction method, it was not used for the pharmacokinetic studies.

Assays. The HPLC assay for carmethizole was carried out using a Hewlett-Packard 1090 HPLC and a 25-cm reversed-phase RP-18 5-μm column (Merck, Darmstadt, FRG) with an elution gradient of 10%–60% acetonitrile in 3 m*M* sodium phosphate buffer (pH 7.2) over 12 min, followed by isocratic elution at 60% acetonitrile in 3 m*M* sodium phosphate buffer (pH 7.2) for 8 min. The flow rate was 1.0 ml/min and the column temperature was maintained at 40° C. Eluting compounds were detected by their UV absorbance at 250 nm on a Hewlett-Packard 798575A variable wavelength detector and peak areas were integrated. Nuclear magnetic resonance (NMR) was conducted with an IBM NR/80 spectrometer. Fast atom bombardment (FAB) mass spectrometry was carried out using a Kratos MS50 mass spectrometer.

Stability studies. The stability of carmethizole in various media was studied by incubating the compound at 25, 50, and 100 μg/ml at 4° C, 23° C (room temperature) and 37° C. Stability was studied over 72 h in 0.9% sodium chloride and 0.1 *M* phosphate buffers, pH 3.0 to 9.0, and over 24 h in fresh citrated human blood, human plasma and dog urine. Samples (0.5 ml) of buffer or biological media were taken for measurement of carmethizole. Plasma protein binding of carmethizole to mouse and human plasma was measured at drug concentrations of 1, 10, and 100 μg/ml using an Amicon 4104 centrifree micropartition system (Amicon, Danvers, Ma, USA) at 23° C. The concentration of carmethizole in the plasma and ultrafiltrate was determined and the percentage plasma protein binding calculated.

Animal studies. In one study, carmethizole was given at a dose of 115 mg/kg (493 mg/m^2) by rapid i.v. injection (<30 s) into the tail vein of unanesthetized male CDF_1 mice weighing 25–30 g that were held in a Broome-type restraint; this dose was optimal for antitumor activity in mice [7]. Blood was collected from groups of three mice at 2, 5, 10, 30, 60, 75, 90, and 120 min; the times represent the midpoints of the blood collection period, which took approximately 30 s. Mice, lightly anesthetized with diethyl ether, were exsanguinated from the retro-orbital venous plexus into heparinized, 1.5 ml centrifuge tubes. The blood was immediately centrifuged at 10,000 *g* for 2 min and 0.2 ml plasma was taken for carmethizole analysis. Urine was also collected from the mice; for each time point, the animals were housed in a glass vessel to collect the spontaneously voided urine. The vessel was washed with water and the washings were combined with the urine remaining in the bladders when the mice were killed. The urine and washings were analyzed for carmethizole.

In another study, male CDF_1 mice weighing 25–30 g were infused through an indwelling PE-10 polyethylene catheter (Intramedic, Clay Adams, Parsepany, NJ) in the tail vein with carmethizole at a rate of 479 μg/min per kg for 8 h, or a total dose of 230 mg/kg (986 mg/m^2). The drug was diluted to 2.38 mg/ml with 0.9% NaCl and infused at 5.1 μl/min. The drug solution was kept at 4° C during the infusion. Plasma collected from groups of three mice killed at 15, 30, 60, 120, 240, 360, and 480 min was subsequently assayed for carmethizole.

A male beagle dog weighing 22 kg was also given carmethizole by i.v. bolus injection over 1 min at a dose of 22.4 mg/kg (493 mg/m^2); this dose was lethal to the animal by day 4. A second male beagle dog weighing 22 kg was given a nonlethal dose of 4.3 mg/kg (99 mg/m^2) carmethizole by i.v. bolus injection. The dogs were placed in a Pavlov-type sling and the drug was injected through a Teflon catheter (Angiocath 18-gauge, Deseet Co., Sandy, Utah, USA) into a cephalic vein. Samples of blood (3 ml) were drawn into a syringe from a second Teflon catheter in the other cephalic vein at 1, 5, 10, 20, 30, 40, 50, 60, 90, 105, 120, 140, 160, and 180 min and immediately transferred to heparinized tubes. Plasma was separated from the whole blood and stored frozen at −20° C overnight before carmethizole analysis.

Pharmacokinetic analysis. Plasma drug-concentration data were subjected to nonlinear least-squares regression analysis using the NONLIN pharmacokinetic computer program [6] with a weighting factor of $1/y^2$. Pharmacokinetic parameters were calculated according to the method of Wagner [10].

Urinary excretion. Carmethizole was given to five CDF_1 mice at a dose of 115 mg/kg (493 mg/m^2) by i.v. bolus injection as described above. The mice were housed in an all-glass metabolism cage and pooled urine was collected for 3 h at 4° C. After 3 h the mice were killed, and the urine in the bladders was collected and combined with the previously excreted urine and water washings from the metabolism cage. Samples (0.05 ml) of the mixture were incubated overnight at 37° C with β-glucuronidase (10,000 units) or aryl sulphatase (700 units) in 0.45 ml 0.1 *M* sodium acetate buffer (pH 5.5). D-Saccharic acid 1,4-lactone (200 m*M*) was added to the aryl sulphatase to inhibit

β-glucuronidase activity [3]. The dogs injected with carmethizole as described above were housed in stainless steel metabolism cages and urine was collected for 24 h at 4° C. Carmethizole and its metabolites in urine were measured by the aforementioned HPLC assay without organic solvent extraction.

In vitro metabolism. Hepatic microsomes and 100,000 *g* cytosol were prepared by differential centrifugation [4] from a homogenate of liver from nonfasted male Fischer 344 rats (Sprague-Dawley, Madison, Wis) in 4 volumes of 0.25 *M* sucrose. The microsomes were washed once in 0.25 *M* sucrose and suspended in 0.1 *M* sodium phosphate buffer (pH 7.4) at a concentration of 16 mg protein/ml. Protein was assayed by the dye-binding method of Bradford [2] using a commercial test kit (Biorad Laboratories, Richmond, Calif) and crystalline bovine serum albumin as a standard.

Incubations contained 6 mg microsomal protein, 1 mmol sodium phosphate buffer (pH 7.4), 75 μmol $MgCl_2$, 6 μmol $NADP^+$, 364 μmol glucose-6-phosphate, 1,051 units glucose-6-phosphate dehydrogenase, and 1 mg carmethizole in a volume of 10 ml. In some incubations microsomes heated to 100° C for 10 min were used. The incubation was conducted at 37° C in a slowly reciprocating, 25-ml Erlenmeyer flask open to the air. Samples of the incubation mixture were taken for carmethizole analysis at 15-min intervals for 2 h.

The reaction between carmethizole and glutathione was studied by incubating 100 μg/ml (0.3 m*M*) carmethizole with 1.0 m*M* glutathione at pH 8.0 for 3 h. Evidence for an enzyme-catalyzed reaction between carmethizole and glutathione was sought by incubating 11 mg 100,000 *g* rat-liver cytosol protein, 1 mmol sodium phosphate buffer (pH 7.4), 30 μmol glutathione, and 1 mg carmethizole in a volume of 10 ml. For blank incubations cytosol was heated to 100° C for 10 min prior to use. The incubation was conducted at 37° C in a slowly reciprocating, 25-ml Erlenmeyer flask open to the air. Samples of the incubation medium were taken for carmethizole analysis at 15-min intervals for 2 h.

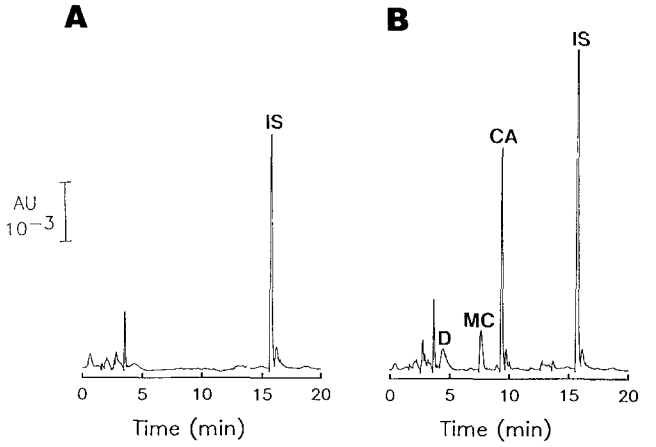

Fig. 2. HPLC chromatogram of plasma from a mouse given 115 mg/kg (493 mg/m^2) carmethizole by i.v. bolus 30 min previously. *A*, blank plasma; *B*, plasma from a mouse given carmethizole. In both cases plasma contained internal standard (*IS*). Peaks: *CA*, carmethizole; *MC*, carmethizole 4′-monocarbamate; *D*, carmethizole diol; *IS*, 4-hydroxybiphenyl internal standard

Results

Assay

The reversed-phase HPLC assay gave good separation of carmethizole and its breakdown products. Ethyl acetate extraction provided a simple way of concentrating carmethizole from biological fluids. The efficiency of drug extraction into ethyl acetate was 86.4% from plasma and 95.3% from 0.9% sodium chloride. A typical chromatogram of carmethizole in mouse plasma is shown in Fig. 2. The HPLC assay with ethyl acetate extraction was linear up to at least 100 μg carmethizole/ml and had a lower detection limit of 0.25 μg/ml for carmethizole in 0.2 ml mouse plasma. The coefficient of variation of the assay for carmethizole in plasma for ten repeated assays was 2% at 100 μg/ml, 4% at 10 μg/ml, 5% at 0.5 μg/ml, and 13% at 0.25 μg/ml.

In vitro stability

The stability of carmethizole was studied in a variety of aqueous media. The drug was found to be relatively stable at 4° C in 0.9% sodium chloride, fresh human plasma, whole human blood, and dog urine with <20% degradation over 24 h (data not shown). At higher temperatures, there was a monoexponential breakdown of carmethizole in these media, with a half-life of ≤1.0 h at 37° C and up to 5.2 h at room temperature (Table 1). The breakdown of carmethizole showed an apparent pH dependency in 0.1 *M* sodium phosphate buffer, with a half-life of 29.0 h at pH 3.0 vs that of 3.4 h at pH 5.0 (Table 1).

Two hydrolysis products of carmethizole were present in 0.9% sodium chloride, one eluting at 7 min and the other, at 4 min. These were identified by comparison with reference compounds as carmethizole 4′-monocarbamate and carmethizole diol, respectively. A third hydrolysis product eluting at 2.5 min was seen only when the drug was incubated in 0.1 *M* sodium phosphate buffer at pH 7.0 or above and not with 0.9% sodium chloride or water (Fig. 3). The NMR spectrum (DMSO or D_2O ppm, delta scale) of

Table 1. Stability of carmethizole in various media

Medium	Half-Life[a]	
	37° C (hr)	Room Temperature (hr)
Sodium chloride, 0.9%	0.67 ± 0.08	3.5 ± 0.5
Sodium phosphate buffer, 0.1 *M*		
pH 3.0	ND	29.0[b]
5.0	ND	3.4
7.0	ND	3.1
9.0	ND	3.1
Whole blood, human	1.00 ± 0.06	5.2 ± 0.25
Plasma, human	0.88 ± 0.06	4.9 ± 0.15
Urine, dog	0.88 ± 0.05	4.4 ± 0.46

[a] Values represeat the mean ± SD of three concentrations of carmethizole (100, 50, 25 μg/ml) measured over 72 h for 0.9% sodium chloride and sodium phosphate buffer and over 24 h for blood, plasma, and urine

[b] A similar half-life was found with 0.1 *M* sodium acetate buffer (pH 3)

ND, not determined

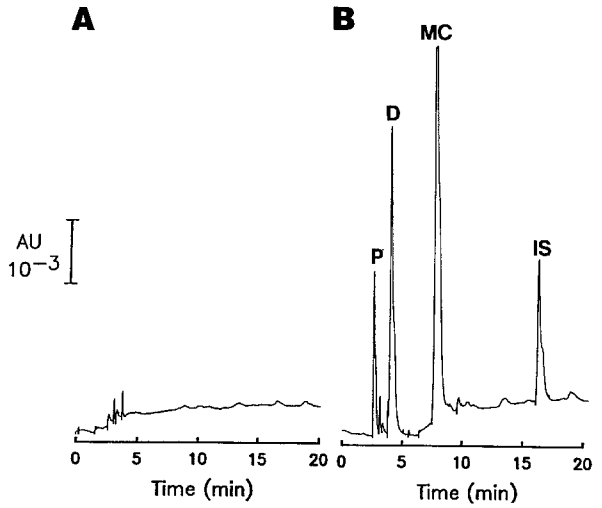

Fig. 3. HPLC chromatrogram of carmethizole added to 0.1 *M* sodium phosphate buffer (pH 5.0) for 4 h at 37° C. The sample was assayed by the direct injection HPLC assay described in the text. *A*, 0.1 *M* sodium phosphate with no drug; *B*, 0.1 *M* sodium phosphate buffer with 100 μg/ml carmethizole. Peaks: *P*, carmethizole diol-4′-monophosphate; *D*, carmethizole diol; *MC*, carmethizole monocarbamate; *IS*, 4-hydroxybiphenyl internal standard

this product showed a downfield shift of the CH_2O protons at 4.4 ppm to 4.7 ppm as a double doublet consistent with a monophosphate ester of carmethizole diol with restricted free rotation. The CH_2O protons of 4.4 ppm were unchanged from the authentic diol. The spectrum showed that the S-CH_3 and N-CH_3 groups had not been hydrolyzed. A contaminant was observed at 1.8 ppm as a singlet whose structure was not determined. The phosphorus-NMR spectrum gave two peaks at 4.5 and 5.6 ppm: that at 4.5 ppm was identified as contaminating phosphate ion, and the 5.6 signal was ascribed to the monophosphate adduct, consistent with that observed for nucleoside monophosphate. The CH_2 group on which the phosphate was located could not be determined by NMR. However, incubation of carmethizole 4′-monocarbamate in 0.1 *M* sodium phosphate buffer (pH 9.0) gave the same carmethizole diol-monophosphate adduct, which identified it as carmethizole diol-4′-monophosphate. In the absence of phosphate ions, the latter slowly hydrolyzed to carmethizole diol. The formation of carmethizole diol-4′-monophosphate was dependent on the pH of the sodium phosphate buffer (Table 2). After 24 h incubation at 37° C in pH 5.0 sodium phosphate buffer, 89% of the carmethizole was converted to carmethizole diol, 2% was present as carmethizole 4′-monocarbamate, and no carmethizole diol-4′-monophosphate was detected. In contrast, after 24 h incubation at 37° C in pH 9.0 sodium phosphate buffer, 84% of the drug was converted to carmethizole diol-4′-monophosphate and 8% was present as carmethizole diol.

Table 3. Plasma protein binding of carmethizole[a]

Sample	Initial concentration (μg/ml)	Percentage of protein bound
Human plasma	100	27%
	10	25%
	1	11%
Mouse plasma	100	28%
	25	30%
	10	27%

[a] Values represent the mean of three separate determinations

Plasma protein binding and red blood cell uptake

Binding of carmethizole to human and mouse plasma protein was determined by ultrafiltration at 23° C (Table 3). The mean plasma protein binding of carmethizole was 21% for human and 28% for mouse plasma. Correction was made for the breakdown of drug during filtration.

The distribution of carmethizole between human red blood cells and plasma was determined by comparing the concentration of drug in fresh whole blood at 37° C with that in plasma separated from the same whole blood. The measurements were made at 30 s, 5 min, and 10 min and extrapolated to zero time. The ratio of carmethizole in blood cells to plasma at a concentration of 100 μg/ml was 0.98 and that at 25 μg/ml, 1.01. The hematocrit of the blood used for the studies was 50%. These results show that carmethizole was not accumulated by red blood cells.

Reaction with glutathione

The ability of carmethizole to react with the cellular nucleophile glutathione was studied by incubating 0.3 m*M* carmethizole with 1 m*M* glutathione at 37° C for 3 h. A product eluting at 3.7 min was seen whose identitiy as the glutathione adduct of carmethizole was confirmed by proton NMR (D_2O). The product showed signals for $CONCH_3$, CH_2S, and CH_2OCO at 2.5, 3.5, and 4.8 ppm,

Table 2. Breakdown products of carmethizole in various media at 37° C[a]

Medium	Percentage of recovery		
	Carmethizole 4-monocarbamate	Carmethizole diol	Carmethizole diol-4-monocarbamate
Sodium Phosphate Buffer, 0.1 M			
pH 5.0	1.6	89.1	0.0
pH 7.0	0.0	44.1	65.7
pH 9.0	0.0	8.0	84.1
Sodium Chloride, 0.9%	0.6	92.9	0.0

[a] Carmethizole (100 μg/ml) was incubated in sodium phosphate buffer or 0.9% NaCl for 24 h at 37° C. The formation of hydrolysis products was determined by direct injection of the medium onto the HPLC. The concentration of carmethizole 4-monocarbamate, carmethizole diol, and carmethizole diol-4-monophosphate was determined by comparison with authentic standards

respectively, consistent with a glutathione adduct of carmethizole monocarbamate. All other signals were consistent with glutathione and carmethizole [5]. FAB mass spectral analysis confirmed the identity of the glutathione adduct of carmethizole monocarbamate, showing a parent ion at m/z 535. Ions were also detected at m/z 557, 579, and 601, corresponding to the addition of 1, 2, and 3 sodiums, respectively. The ion formed from the loss of glutathione, m/z 228, was also present in the spectrum. The other fragment ions were consistent with a glutathione adduct of carmethizole monocarbamate. The fragmentation pattern did not allow assignment of the glutathione to the C-4′ or C-5′ carbon. No conjugate was formed when glutathione was incubated with carmethizole 4′-monocarbamate. It appears that the glutathione must react directly with carmethizole and form, most probably, carmethizole 4′-monocarbamate-5′-glutathione.

In vitro metabolism

When carmethizole was incubated with either hepatic 100,000 *g* cytosol in the presence of glutathione or microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), it had a half-life of 0.67 h. The half-life of carmethizole with heat-inactivated cytosol or microsomes was not significantly different, at 0.63 h. After the 2 h incubation with hepatic microsomes or 100,000 *g* cytosol, only 33% ± 4.6% of the carmethizole initially present could be recovered, 13% as unchanged drug and 20% as carmethizole 4′-monocarbamate. Heat inactivation of the microsomes and cytosol did not affect this recovery. Carmethizole appears to react directly with the protein present in the incubation medium.

Disposition and pharmacokinetics

Mouse. Plasma concentrations of carmethizole following an i.v. bolus dose of 115 mg/kg (493 mg/m^2) in the mouse are shown in Fig. 4. The elimination of drug from the plasma was best fit by a one-compartment open model; the pharmacokinetic parameters are shown in Table 4. The highest concentration of carmethizole measured in plasma was 160 μg/ml at 2 min after i.v. injection; at 120 min, the concentration was below the limit of detection. Two breakdown products, carmethizole 4′-monocarbamate and carmethizole diol, were detected in mouse plasma (Fig. 2). Both compounds extracted poorly into the ethyl acetate,

Table 4. Pharmacokinetic parameters of carmethizole given by i.v. bolus to mouse and dog[a]

	Mouse (493 mg/m^2)	Dog 1 (493 mg/m^2)	Dog 2 (99 mg/m^2)
Half-life			
alpha (min)		0.9	3.7
beta (min)	11.6	39.2	46.3
V_d (ml/kg)	728.9	242.5	448.8
Cl (ml/min per kg)	43.6	4.6	7.7

[a] Carmethizole was given by i.v. bolus to mice and beagle dogs at the doses indicated in parentheses. Pharmacokinetic parameters for carmethizole in plasma were determined from the data in Fig. 4 and 6. V_d, apparent volume of distribution; Cl, total body plasma clearance

such that quantification of the breakdown products was not possible.

Plasma concentrations of carmethizole in male CDF_1 mice infused with 230 mg/kg (986 mg/m^2) over 8 h are shown in Fig. 5. A steady-state plasma concentration of 11.7 μg/ml was achieved within 180 min of infusion; the calculated total body clearance of drug was 40.8 ml/min per kg, or 175 ml/min per m^2. This compares with a total body clearance of 43.6 ml/min per kg, or 187 ml/min per m^2, following an i.v. bolus and a predicted steady-state plasma concentration of 11 μg/ml carmethizole for the infusion rate used.

Dog. Carmethizole was given to a male beagle dog at the same dose on a surface area basis as in the mouse, that is, 493 mg/m^2 (22.4 mg/kg). The dog became extremely agitated at approximately 25 min postinjection and remained so until approximately 2 h postinjection. It was possible to conduct the pharmacokinetic study. The animal experienced severe vomiting for 24 h postinjection and died during the night 4 days later. Postmortem examination revealed an apparently normal liver but major intestinal bleeding.

Pharmacokinetic studies were conducted in another beagle dog at a lower, nonlethal dose of 4.3 mg/kg (99 mg/m^2) carmethizole. The elimination of drug from dog plasma at both doses was best described by a two-compartment open model (Fig. 6). Pharmacokinetic pa-

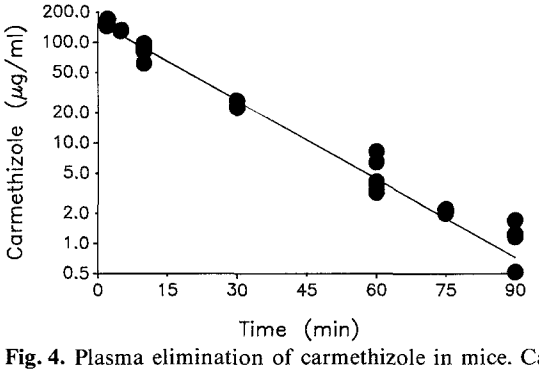

Fig. 4. Plasma elimination of carmethizole in mice. Carmethizole was given to mice at a dose of 115 mg/kg (493 mg/m^2) by i.v. bolus. Each point represents one animal. The *continuous line* is the computer fit to the data, $r = 0.93$

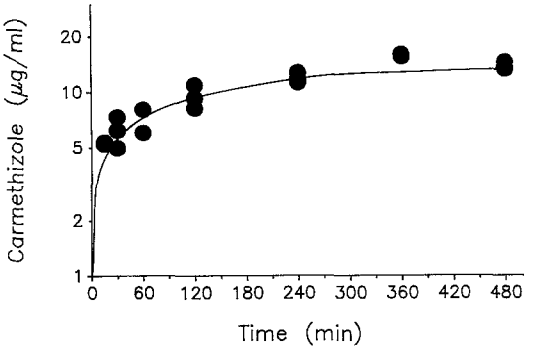

Fig. 5. Plasma carmethizole in mice receiving a continuous i.v. infusion of carmethizole at 230 mg/kg (986 mg/m^2) over 8 h. Each point represents one animal

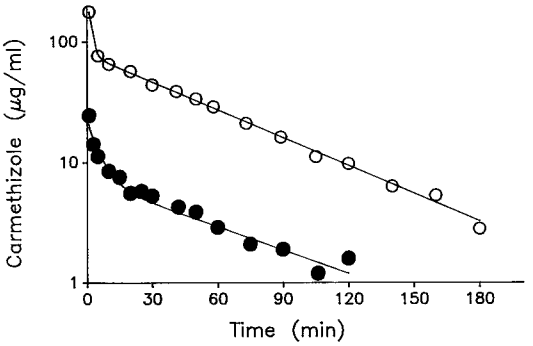

Fig. 6. Plasma elimination of carmethizole from beagle dogs. Carmethizole was given to the dogs at a dose of (O) 493 mg/m^2 or (●) 99 mg/m^2. The dog receiving 493 mg/m^2 died on day 4. The *continuous lines* are the computer fit to the data

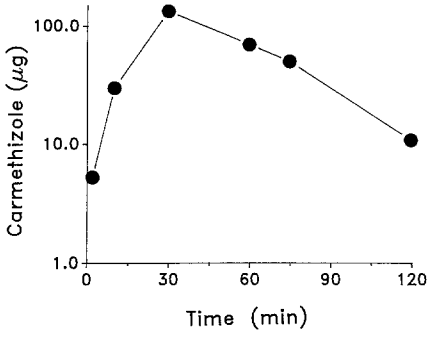

Fig. 7. Urinary excretion of carmethizole in mice. Groups of three mice were given carmethizole at 115 mg/kg (493 mg/m^2) by i.v. bolus. Each point is the excretion of carmethizole in the pooled urine

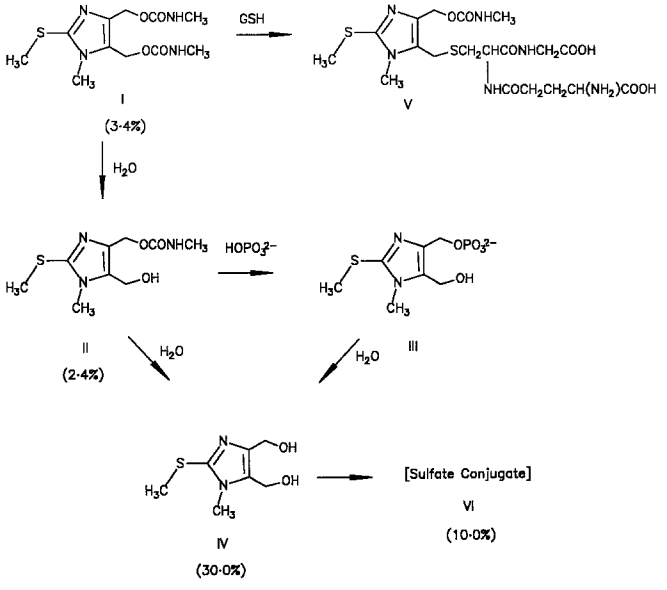

Fig. 8. Pathway for the formation of metabolites and breakdown products of carmethizole. Structures: *I*, carmethizole; *II*, carmethizole 4′-monocarbamate; *III*, carmethizole diol-4′-monophosphate; *IV*, carmethizole diol; *V*, carmethizole monocarbamate glutathione adduct (the specific carbamate group displaced by glutathione has not been determined, but the most likely structure is shown); *VI*, sulfate conjugate of carmethizole diol. Values in parentheses represent the percentage excreted in mouse urine over 3 h

rameters for the two dogs are shown in Table 4. The terminal half-life was 39.2 min at a dose of 22.4 mg/kg and 46.3 min at 4.3 mg/kg. The dog receiving 22.4 mg/kg had a total body clearance of 4.6 ml/min per kg, whereas that in the animal given 4.3 mg/kg was 7.7 ml/min per kg.

Urinary excretion

Mice given carmethizole by i.v. bolus excreted most of the unchanged drug in the urine in the first 30 min (Fig. 7); the mean renal clearance over 30 min was 0.9 ml/min per kg. In all, 46% of the carmethizole dose was excreted in the urine over 3 h as unchanged drug or drug-derived products. The major urinary product was carmethizole diol, which accounted for 30% of the drug given; unchanged carmethizole and carmethizole 4′-monocarbamate accounted for 3.4% and 2.4%, respectively. Incubation of the urine with aryl sulfatase for 24 h resulted in the appearance of a peak corresponding to carmethizole diol and an equivalent decrease in a peak at 3 min (data not shown). This sulfate conjugate of carmethizole diol accounted for 10.0% of the drug given. Incubation of the urine with β-glucuronidase for 24 h did not increase the recovery of carmethizole or any of its breakdown products. The pathways for the urinary excretion of carmethizole and its reaction products in the mouse are shown in Fig. 8. The urine collected from dogs following the i.v. bolus administration of carmethizole was contaminated with vomit and was thus unsuitable for direct injection onto the HPLC.

Discussion

Carmethizole is relatively unstable in aqueous media, having a half-life of $\leqslant 1$ h in 0.9% sodium chloride, human whole blood, human plasma, and dog urine at 37° C. Its breakdown showed an apparent pH dependency in sodium phosphate buffer, being more stable below its pKa value of 5 [1]. It has been predicted that protonated carmethizole should be more stable than the free base because the production of charged hydrolysis intermediates is more difficult if the ring is already protonated [1]. The major breakdown product of carmethizole in aqueous media was found to be carmethizole diol, which is probably formed through the carmethizole 4′-monocarbamate intermediate. Anderson et al. [1] have studied the hydrolysis of carmethizole in aqueous solution and reported that the hydrolysis intermediate was carmethizole 4′-monocarbamate rather than carmethizole 5′-monocarbamate. The two carmethizole monocarbamates can be separated by HPLC [1, 7]. Although carmethizole 5′-monocarbamate reference compound was not available, in all of our studies we never saw a breakdown product HPLC peak that might be attributed to carmethizole 5′-monocarbamate.

A third product was seen when carmethizole was incubated in 0.1 *M* sodium phosphate buffer at neutral or alkaline pH; this compound was identified as a monophosphate adduct of carmethizole diol by proton and phosphorus NMR. The position of the phosphate group could not be identified by NMR but, because the phosphate adduct was also formed from carmethizole 4′-monocarbamate, it was most likely carmethizole diol-4′-monophosphate. The pH dependency of carmethizole diol-4′-monophosphate formation is most likely related to the pKa of phosphate, as the latter is a better nucleophile under alkaline conditions. The in vivo significance of the carmethizole diol-

phosphate adduct is not known. We could not tell if this adduct was present in urine because of contaminating background peaks. Although the carmethizole diol-phosphate adduct lacks both carbamate groups, the phosphate is a possible leaving group and, if formed within cells, the adduct might act as an alkylator. It should be noted that another antitumor alkylating agent, mitomycin C, has been reported to undergo spontaneous [8] as well as enzymatic [9] activation to a species that reacts with phosphate to form phosphate adducts.

Carmethizole was also found to react directly with glutathione. The glutathione adduct of carmethizole monocarbamate was identified by proton NMR and FAB mass spectrometry. The fragmentation pattern did not allow the assignment of glutathione to the C-4′ or C-5′ carbon, but the reactivity of the 5′ carbamate group suggests that this is the most likely position for attack by glutathione. The glutathione adduct retained a carbamate group and could therefore still act as an alkylator. Again, we could not tell if the carmethizole glutathione adduct was present in urine because of contaminating background peaks.

Carmethizole given by i.v. bolus to mice exhibited rapid monophasic elimination from the plasma, with a half-life of 11.6 min. The in vitro half-life of carmethizole in whole blood was 60 min at 37° C. Thus, the drug was eliminated more rapidly from the plasma than would be expected if the elimination were solely due to chemical breakdown. The more rapid chemical degradation in vivo might represent the reaction of carmethizole with endogenous compounds, for example, glutathione. Following the i.v. bolus administration of carmethizole, the plasma and urine of mice contained only unchanged drugs, carmethizole 4′-monocarbamate, and carmethizole diol, together with a small amount of the sulfate conjugate of carmethizole diol in urine. No other metabolic products were detected, although without radiolabeled drug it is not possible to say whether other products might have been present in the urine but obscured by endogenous peaks.

Carmethizole was given by i.v. bolus to beagle dogs at doses of 493 and 99 mg/m^2. The elimination of drug from the plasma of the beagle dog was slower than that in the mouse and was biphasic. The terminal phase half-life was 43 ± 5.0 min at both doses in the beagle dog, whereas the mouse plasma-elimination half-life was 11.6 min. Carmethizole appears to be more toxic to dogs than to mice; the compound was lethal to the beagle dog at a dose that was nontoxic to the mouse, that is, 493 mg/m^2. The increased toxicity of carmethizole in the dog might be due to its slower elimination in this animal. The dog receiving 493 mg/m^2 carmethizole had a total body clearance that was 60% of the total body clearance determined for the dog receiving 99 mg/m^2. This could indicate the saturation of an elimination process in the dog, which cannot be confirmed with only two dogs. The elimination processes for carmethizole were not saturated in the mouse since the total body clearance following i.v. bolus administration did not differ significantly from that with an 8-h infusion. The plasma from the beagle dog contained the same breakdown products as mouse plasma, suggesting that carmethizole is eliminated by the same pathways in the dog as in the mouse and that toxicity is not related to the formation of a unique metabolite.

In summary, carmethizole was found to be relatively rapidly broken down to carmethizole diol in 0.9% sodium chloride and biological media. When the drug was added to phosphate buffer at neutral or basic pH, it also formed carmethizole diol-4′-monophosphate; it reacted directly with glutathione, forming a glutathione adduct of carmethizole monocarbamate. Carmethizole was rapidly eliminated from the plasma of mice following i.v. bolus administration but was more slowly eliminated from dog plasma. The major product formed in vivo was carmethizole diol, most likely by spontaneous degradation.

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