ACKNOWLEDGMENT

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Urinary Metabolites of Cambendazole

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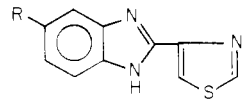
Metabolism of orally administered cambendazole-^{14}C [isopropyl 2-(4-thiazolyl)-5-benzimidazolecarbamate] in cattle, swine, and sheep leads to urinary excretion of 20–40% of the dose as biotransformation products. Fourteen urinary metabolites were identified, primarily by mass spectrometric techniques, and eleven of these were synthesized. Metabolic attack on the thiazole ring is the major route of structural transformation, resulting in a series of 11 identified metabolites. The carbamate side chain is also involved in several metabolic changes, and hydroxylation of the benzene ring yielded a phenolic metabolite.

Cambendazole, isopropyl 2-[4-thiazolyl]-5-benzimidazolecarbamate (CBZ), is a broad-spectrum anthelmintic active in cattle, swine, and sheep against a wide variety of mature and immature parasites (Hoff et al., 1970; Campbell and Yakstis, 1970; Egerton and Campbell, 1970; Egerton et al., 1970a,b). This drug is a substituted thiabendazole (TBZ) (Brown et al., 1961). TBZ is also an anthelmintic and antifungal agent (Campbell, 1961; Cuckler, 1961; Robinson et al., 1964). Both drugs are rapidly metabolized after oral administration to test animals. Tocco et al. (1964) reported on the metabolic fate of TBZ in the sheep and indicated that the phenol 5-hydroxy-TBZ and its glucuronide and sulfate conjugates were urinary metabolites in this species. Hydroxylation at this position of the benzimidazole ring system followed by conjugation is a metabolic pathway for 2-(2-furyl)-benzimidazole (Frank, 1971) and benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] (Gardiner et al., 1974). Tyler et al. (1976) have demonstrated that in the rat, hydroxylation at the 5-position is the preferred route of metabolism for benzimidazole itself. When the 5-position of the benzimidazole nucleus of a drug is substituted, metabolic reactions may occur elsewhere on the molecule. Parbendazole (methyl 5-butyl-2-benzimidazolecarbamate), for example, is metabolized by sheep and cattle to form a series of oxidative products of the butyl side chain (Dunn et al., 1973) and we (VandenHeuvel et al., 1972) have reported that CBZ is transformed to several side chain metabolites in various species. It was thus of interest to further investigate the urinary metabolite pattern for CBZ so as to ascertain the positions of metabolic attack in this 5-substituted benzimidazole, especially with respect to the thiazole ring.



CBZ, R = $(CH_3)_2CHOCHN$; TBZ, R = H; 5-HTBZ, R = OH

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EXPERIMENTAL SECTION

Radiochemical Procedures.. *Tracers Employed.* Radioactively labeled CBZ was employed in the metabolism studies; for the syntheses of the labeled compounds [CBZ-^{14}C (benzene ring, 0.15–1.2 μCi/mg) and CBZ-*t* (isopropyl methyl, 6.0 μCi/mg)], see Ellsworth et al. (1976). Each compound exhibited (by thin-layer chromatography) >99% radiochemical purity.

Liquid Scintillation Counting. Measurements of radioactivity were made using either a Packard Tri-Carb or an Intertechnique liquid scintillation spectrometer operated on optimal setting for ^{14}C or ^{3}H. Scintillation solutions were made from either Omnifluor or Liquifluor (Bio-Rad) in toluene or a 70:30 mixture of toluene–ethanol. When required, quenching corrections were made by the internal standard method using the appropriate standard. Samples insoluble in the phosphor were analyzed by combustion to $^{14}CO_2$ or tritium water using the Schoniger method (Kelly, 1961) or the more rapid Peterson (1969) modification.

Animal Studies. Large animals were kept in metabolism stalls or cages so that feces and urine could be collected separately. The single dose of drug was administered either as a dry blend (or gelatin capsule) or in

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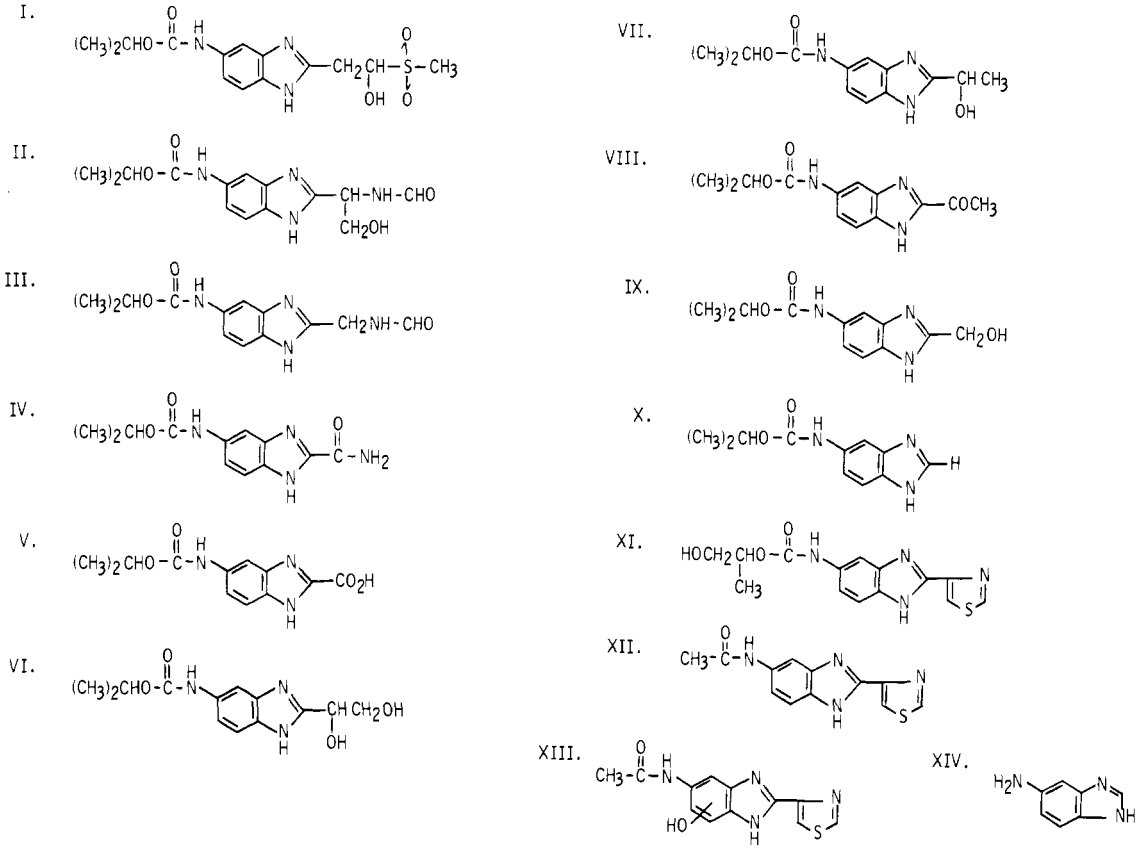
Table I. Structure of Cambendazole Metabolites



solution (ethanol or polyethylene glycol). Urine (0–24 h post dose) was filtered through spunglass wool at the time of collection. The samples were frozen until used for radiochemical assay and metabolite isolation.

Isolation of Metabolites from Urine. The metabolites in urine were isolated by a series of extraction steps. The urine samples were extracted twice with two equal volumes of methylene chloride, first at pH 3.5 and subsequently at pH 7. The resulting aqueous fraction was then extracted at pH 7 twice, with two equal volumes of ethyl acetate followed by two equal volumes of 1-butanol. The crude fractions thus obtained were used for further purification by chromatographic methods, including chromatography on XAD-2 resin. Thin-layer chromatography was used extensively to monitor the fractionation procedures and for the final purification prior to the spectral examination. All of the isolated metabolites were obtained from the ethyl acetate fraction except V and X (1-butanol fraction) and VIII (XAD-2 chromatography of urine). Based on radioactivity, ~20–40% of the oral doses were found in 0–24 h urines; ~30–35% of this radioactivity was extractable using methylene chloride and ethyl acetate, and another ~30% was extractable with 1-butanol. CBZ was the only compound identified in the methylene chloride fractions. Most of the metabolites were found to be common to all animal species investigated.

XAD-2 Resin Treatment. XAD-2 resin (Rohm & Haas Co.) adsorbs compounds from aqueous solutions. After thorough washing with water to remove polar impurities, the adsorbed compounds were removed by elution with water–methanol mixtures or with water saturated with ethyl acetate.

Thin-Layer Chromatography. Samples were prepared for thin-layer chromatography by evaporation of a suitable aliquot, and the residue was redissolved in methanol for application. Analtech silica gel (GF) plates were used. Solvent systems commonly employed were: (I) benzene–dioxane–concentrated NH_4OH, 10:80:10; (II) 1-butanol–water–acetic acid, 65:25:10; (III) benzene–ethanol, 80:20.

Thin-layer plates were examined visually for fluorescence quenching and scanned for radioactive zones using a Packard Radiochromatogram scanner No. 7201. Quantitative measurement was achieved by scraping the layer from the plate using an Analabs Zonal Scraper which collects the adsorbent in Packard counting vials, or by scraping selected sections into counting vials. One milliliter of methanol followed by 10–15 mL of 70:30 scintillation mixture was added to each vial prior to counting.

Radioautography of Urine Samples. For comparison of the number and general character of the CBZ metabolites in the urine of the various test animals a two-dimensional thin-layer chromatography system involving radioautography (Jacob et al., 1975) was devised to separate the urinary metabolites.

Gas–Liquid Chromatographic and Mass Spectrometric Techniques. Combined gas–liquid chromatography–mass spectrometry (GLC–MS) and direct probe MS were carried out with an LKB Model 9000 instrument. Spiral glass columns 90–180 cm × 3 mm i.d. packed with 1% stationary phases (SE-30, OV-1, or OV-17) were employed. Column temperatures were from 190 to 255 °C, with a helium flow rate of 30 mL/min. Direct probe temperatures were between 100 and 200 °C. Typically, the probe temperature was increased slowly to volatilize those sample components of relatively high vapor pressure; CBZ-related compounds usually required significantly higher probe temperatures, and thus a purification was effected. High-resolution MS was carried out with a CEC-21-110 instrument via the peak matching technique (perfluorokerosene as reference standard). Gas–liquid radiochromatography was carried out using a Barber-

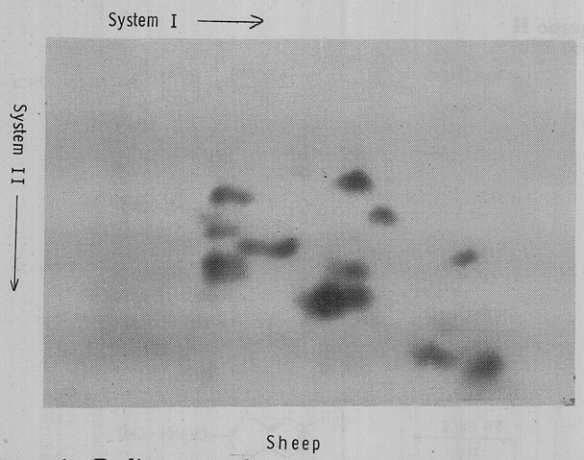


Figure 1. Radioautograph of two-dimensional TLC of ethyl acetate extract from urine of sheep given CBZ-^{14}C.

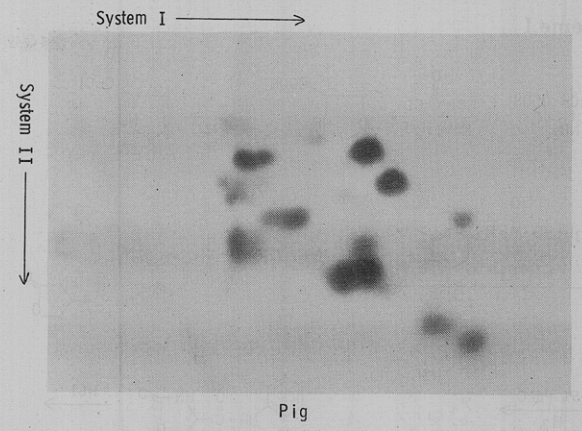


Figure 2. Radioautograph of two-dimensional TLC of ethyl acetate extract from urine of pig given CBZ-^{14}C.

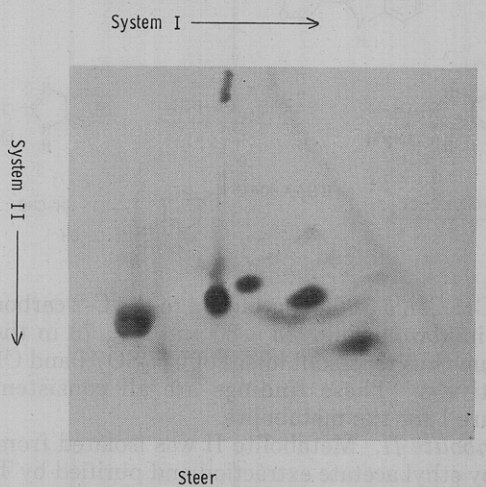


Figure 3. Radioautograph of two-dimensional TLC of ethyl acetate extract from urine of steer given CBZ-^{14}C.

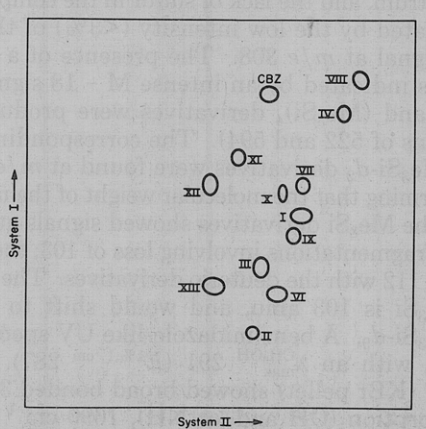


Figure 4. Depiction of two-dimensional TLC showing position of principal CBZ metabolites.

Colman Model 5000 instrument. This exit gas was split and part was directed through an FID detector and the remaining fraction was analyzed for radioactivity using either a radioactivity monitor or a collection manifold (Trenner et al., 1972).

The mass spectrometric behavior of CBZ has been reported (VandenHeuvel et al., 1972). Prominent fragment ions are M − 42, m/e 260 (loss of C_3H_6 from the isopropoxycarbonylamino group); M − 60, m/e 242 (elimination of the elements of 2-propanol); and M − (42 + 44), m/e 216 (loss of C_3H_6 and CO_2). Pairs of signals 42 mass units apart (i.e., M and M − 42) proved especially useful for characterizing a spectrum from an isolate as arising from a CBZ metabolite possessing the intact isopropoxycarbonylamino side chain. The parent drug forms a bis(trimethylsilyl) (Me_3Si) derivative with intense fragment ions at M − 42 and M − 132 (loss of $C_3H_7OMe_3Si$).

CBZ and related compounds possessing the isopropoxycarbonylamino side chain can be eluted intact and as a single peak when subjected to GLC at <200 °C. At higher temperatures the corresponding isocyanate, resulting from thermal elimination of 2-propanol, is also observed. The major fragment ion for the isocyanate is M − 27, loss of HCN from the thiazole ring. GLC of trimethylsilylated CBZ results in the elution of only one compound, the Me_3Si isocyanate, molecular weight 314; injection port (and/or on-column) thermal transformation to the isocyanate is more facile with the Me_3Si derivative than with the parent drug.

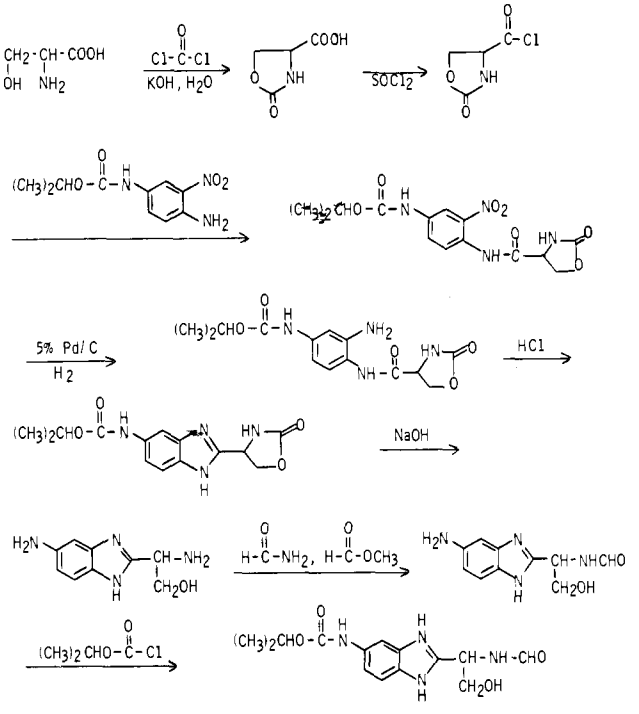
Preparation of Derivatives. Me_3Si and Me_3Si-d_9 derivatives were prepared by dissolving ~10 μg of the metabolite in 20 μL of a 2:1 bis(trimethylsilyl)acetamide (or bis(trimethylsilyl)acetamide-d_{18})–pyridine solution and heating at 50 °C for 15 min. After derivatization, mass spectra were obtained by GLC–MS or direct probe.

RESULTS

Identification of Metabolites. *Comparison of Animal Metabolites.* Photographs of the radioautographs of two-dimensional thin-layer chromatography plates (analyses of ethyl acetate extracts of sheep, pig, and steer urines) are reproduced in Figures 1–3, respectively: a drawing showing the positions of the major isolated metabolites is also given (Figure 4). The origin in each chromatogram was in the upper left-hand corner of the plate.

Metabolite I. Metabolite I was isolated from ovine urine by ethyl acetate extraction and purified by TLC on silica gel (R_f 0.5 in system I; R_f 0.7 in system II). High-resolution MS indicated an empirical formula of $C_{14}H_{19}O_5N_3S$ (calcd, 341.1044; found, 341.1058). The presence of sulfur was confirmed by the intensity of the M + 2 isotope signal at m/e 343. Fragment ions were observed at M − 18 (loss of water), M − 42 (loss of C_3H_6), M − 59, and M − 60 (losses of C_3H_7O and C_3H_7OH, respectively), M − 80, M − 79, and M − (80 + 42). The infrared spectrum (KBr pellet) contained absorption bands characteristic of sulfonyl (1130, 1290 cm^{-1}), hydroxyl (3300 cm^{-1}), and carbonyl (1690 cm^{-1}; entirely in line with the presence of the isopropoxycarbonylamino side chain). The UV spectrum was benzimidazole-like, with $\lambda_{max}^{CH_3OH}$ 290 ($E^{1\%,1\ cm}$ 268). The NMR spectrum (0.1 N DCl) was consistent with the presence of methanesulfonyl (δ 3.13), isopropoxycarbonyl [$HC(CH_3)_2$, δ 4.91)], and a side chain -$CH_2CH(X)(Y)$ [CH_2CH, δ 3.97,

Scheme I

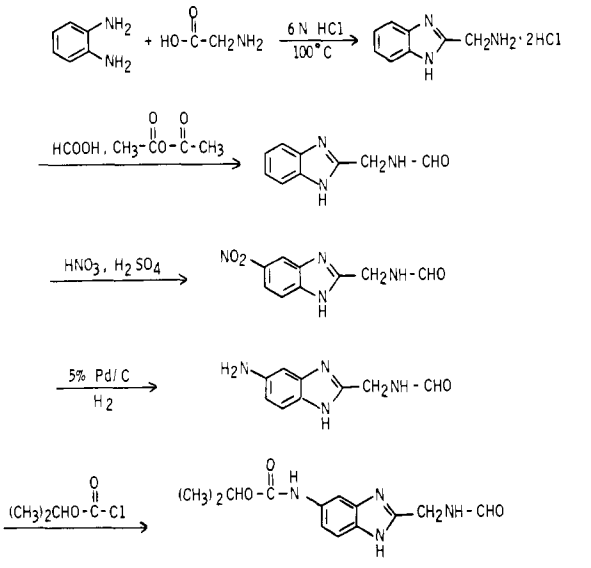


d and CH_2CH, δ 5.86, tr] attached to the C-2 carbon atom of the imidazole ring. M − 80 and M − 79 in the mass spectrum thus represent losses of CH_3SO_2H and CH_3SO_2, respectively. These findings are all consistent with structure I for the metabolite.

Metabolite II. Metabolite II was isolated from ovine urine by ethyl acetate extraction and purified by TLC on silica gel (R_f of 0.2 in system I; R_f of 0.6 in system II). An apparent molecular ion was observed at m/e 306 in the mass spectrum, and the lack of sulfur in the compound was demonstrated by the low intensity (<3%) of the M + 2 isotope signal at m/e 308. The presence of a hydroxyl group was indicated by an intense M − 18 signal. Both $(Me_3Si)_3$ and $(Me_3Si)_4$ derivatives were produced (molecular ions of 522 and 594). The corresponding signals for the Me_3Si-d_9 derivatives were found at m/e 549 and 630, confirming that the molecular weight of the metabolite is 306. The Me_3Si derivatives showed signals at m/e 103 and also fragmentations involving loss of 103; these values shifted to 112 with the deuterio derivatives. The fragment CH_2OMe_3Si is 103 amu, and would shift to 112 with CH_2OMe_3Si-d_9. A benzimidazole-like UV spectrum was observed, with an $\lambda_{max}^{CH_3OH}$ 291 ($E^{1\%,1\ cm}$ 287). The IR spectrum (KBr pellet) showed broad bonded 3500–2700 cm^{-1} absorption (OH and/or NH), 1690 cm^{-1} (isopropoxycarbonylamino), and 1630 cm^{-1} (formamidocarbonyl). These data suggest the presence of a hydroxymethyl group in the metabolite, and structure II was proposed. The racemic compound was synthesized and found to possess the same spectrometric (mass and IR) and TLC properties as the metabolite. The abbreviated synthesis scheme is indicated in Scheme I.

Metabolite III. Metabolite III was isolated by ethyl acetate extraction of bovine urine and purified by TLC on silica gel (R_f values of 0.45, 0.6, and 0.5 with solvent systems I–III, respectively). A molecular weight of 276 was suggested by MS. The mass spectrum showed a signal at M − 42; M − 29 was also noted—possibly loss of CHO. The low relative intensity of the M + 2 signal (m/e 278), 2%, indicated the absence of a sulfur atom. MS of the trimethylsilylated isolate gave a new molecular ion at m/e 492, an increase of 216 mass units. This can be accounted for by the formation of a $(Me_3Si)_3$ derivative [276 + (3 × 72) = 492]. An intense fragment ion was found at M − 132; this is the loss of $C_3H_7OMe_3Si$, characteristic of compounds (such as CBZ) which possess a trimethylsilylated isopropoxycarbonylamino group. Derivatization with BSA-d_{18} to form the Me_3Si-d_9 derivative resulted in a shift in the molecular ion from m/e 492 to 519, an increase of 27 amu, confirming the presence of three derivatizable groups in the metabolite.

Scheme II



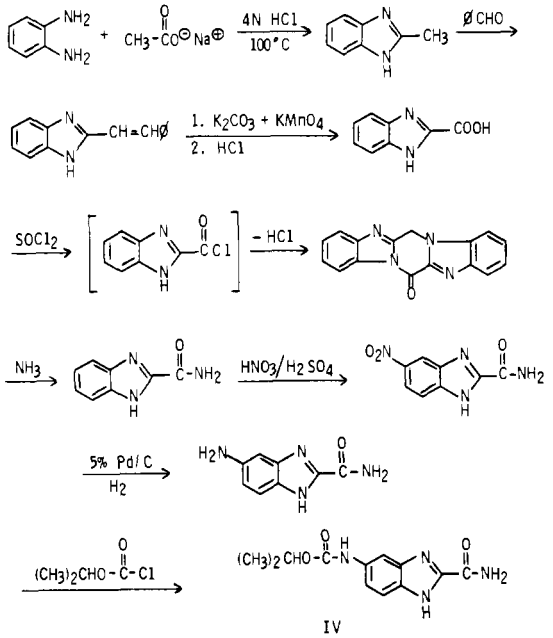
On the basis of these data, a structure was proposed with the substituent C_2H_4NO, a moiety containing one derivatizable group, on the 2-position of the benzimidazole. A number of possible structures for this substituent were considered, including $CH_2NHCH(=O)$. Several model compounds were examined, and one containing the formamide moiety was found to exhibit an M − 29 fragment. Structure III was then synthesized and found to possess the same mass spectrometric and TLC behavior as that of the metabolite.

The synthetic route is indicated in Scheme II.

Metabolite IV. Metabolite IV was isolated by ethyl acetate extraction of ovine urine and purified by TLC on silica gel; the R_f values were 0.8, 0.8, and 0.65 for solvent systems I–III, respectively. The mass spectrum of a TLC isolate contained many signals, including those at m/e 262, 220, and 203. As CBZ and those metabolites which contain the intact isopropoxycarbonylamino side chain characteristically exhibit M − 42 fragment ions, it was tentatively assumed that the spectrum from the isolate did indeed contain a side chain intact CBZ metabolite of molecular weight 262. The even molecular weight required that the metabolite contain an even number of nitrogen atoms, and a possible structure was that of the amide IV.

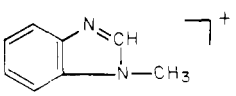
Earlier work with the photolysis of TBZ had demonstrated that the mass spectrum of benzimidazole-2-carboxamide contains an M − 17 fragment ion (Jacob et al., 1975). The presence of an M − (42 + 17) fragment in the metabolite spectrum supported the proposed structure. The isolate was subjected to trimethylsilylation conditions, and the appropriate molecular ion shift (to m/e 550) was observed. The authentic carboxamide was prepared and shown by MS and TLC techniques to be identical with the metabolite. A study (VandenHeuvel et al., 1974) employing CBZ-*thiazole*-^{15}N has demonstrated that in the rat the carboxamide N arises from the thiazole ^{15}N. The synthesis is outlined in Scheme III.

Scheme III



IV

Metabolite V. An acidic metabolite isolated by 1-butanol extraction of ovine urine was purified by TLC on silica gel. An R_f of 0.5 was found with the developing solvent chloroform–methanol–water–concentrated NH_4OH (55:35:6:4). The solvent system benzene–dioxane–NH_4OH (50:50:1) gave an R_f of 0.6; an *Rf* of 0 was observed using solvent system I. V was found to produce a mass spectrum (direct probe) indistinguishable from that of 5-isopropoxycarbonylaminobenzimidazole (vide infra, metabolite X). TLC evidence indicated that metabolite V is converted to metabolite X upon standing in solution. MS of benzimidazole-2-carboxylic acid (mol wt 162) resulted in a spectrum identical with that of benzimidazole (mol wt 118). Methylation of benzimidazole-2-carboxylic acid with diazomethane in methanol resulted in conversion to the *O,N*-dimethyl derivative, molecular weight 190. The major fragment ion in the spectrum of this compound is M – 58, presumably loss of CO_2CH_2 by a cyclic mechanism.

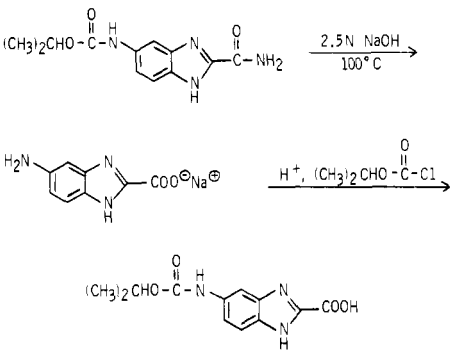


M-58

Methylation of the metabolite under the same conditions resulted in formation of a new compound with an apparent molecular ion of *m/e* 291 and intense fragment ions at *m/e* 249 (M – 42) and 191 [M – (42 + 58)] plus less intense ions [M – (42 + 44); M – (42 + 45)] characteristic of the isopropoxycarbonylamino function. Room temperature trimethylsilylation produced a compound with a molecular ion of *m/e* 335, and intense fragment ions of *m/e* 293 and 278 [M – 42 and M – (42 + 15)]. Some evidence was also observed for the decarboxylated metabolite. These data strongly suggested that the metabolite possessed the structure of the carboxylic acid V. This compound was synthesized as outlined in Scheme IV. The mass spectrometric (free, methylated, and trimethylsilylated) and TLC (free) behavior of the metabolite and the synthetic compound were identical.

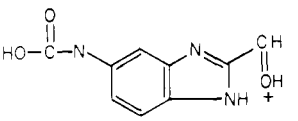
Metabolite VI. Metabolite VI, isolated from ovine urine by ethyl acetate extraction, was purified on silica gel. When developed with solvent system I, an R_f of 0.2 was found; with systems II and III, R_f values of 0.6 and 0.3 were noted. VI was found to possess a molecular weight of 279

Scheme IV



by direct probe MS. Fragment ions were observed at *m/e* 261 (M – 18), 248 (M – 31), 247 (M – 32), 237 (M – 42) 219 (M – 60), 206 [M – (42 + 31)], and 162 [M – (42 + 44 + 31)]. Several of these ions indicate the presence of the isopropoxycarbonylamino side chain in the molecule. Further, loss of 18 suggests the presence of a hydroxyl group. Attempts to analyze the metabolite by gas–liquid radiochromatography were unsuccessful—no radioactive peak could be obtained, suggesting that the compound possesses considerable polarity. A possible structure, based on a molecular weight of 279, was the glycol VI.

Losses of 42 and 60 amu can be accounted for by fragmentation involving the isopropoxycarbonylamino group, and losses of 18 and 31 can be accommodated by elimination of water and CH_2OH, respectively, from the glycol side chain. The fragment of *m/e* 206 [M – (42 + 31)], for example, can be ascribed to the structure:



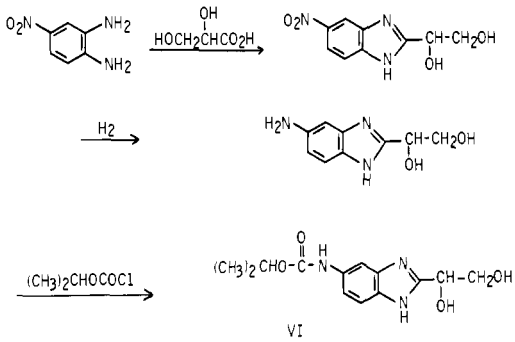
m/e 206

Oxidation of the metabolite with periodate produced a new substance which possessed a molecular ion of *m/e* 247, 32 amu less than the metabolite. Fragmentation attributable to the isopropoxycarbonylamino group (e.g., M – 42) was observed, but no loss of 31 amu. The oxidation product formed an *O*-methyloxime, molecular ion *m/e* 276; the main fragment ion found for this derivative is *m/e* 234, M – 42. It is thus likely that the oxidation product is an aldehyde. These data strongly support the suggested glycol structure of the metabolite.

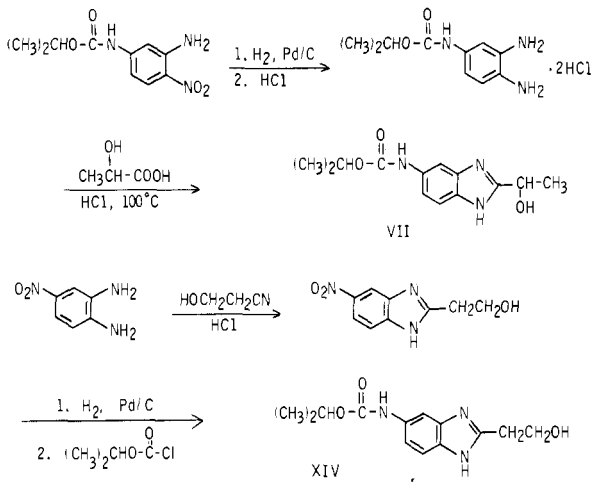
The mass spectrum (direct probe) of an aliquot of the metabolite subjected to trimethylsilylation (BSA–pyridine, 50 °C, 0.5 h) indicated the presence of four active hydrogen atoms in the metabolite. The molecular ion of the derivative is found at *m/e* 567 [279 + (4 × 72)]. The derivative gave an intense fragment ion at *m/e* 464, M – 103 (CH_2OMe_3Si). Loss of 132 amu ($C_3H_7OMe_3Si$) confirms the presence of the isopropoxycarbonylamino group in the metabolite, as this fragmentation is found to occur with the Me_3Si derivative of those compounds possessing this moiety. The metabolite structure was thus established as the glycol VI. This was confirmed by mass spectrometric and TLC comparison with a synthetic sample of the racemic glycol prepared as outlined in Scheme V.

Metabolite VII. Metabolite VII, isolated from porcine urine by ethyl acetate extraction, was purified by TLC on silica gel; in systems I and II it exhibited R_f values of 0.6 and 0.7, respectively. Its apparent molecular weight was 263 as determined by MS, and it exhibited a fragmentation pattern compatible with the presence of the intact isopropoxycarbonylamino side chain. The low intensity

Scheme V



Scheme VI

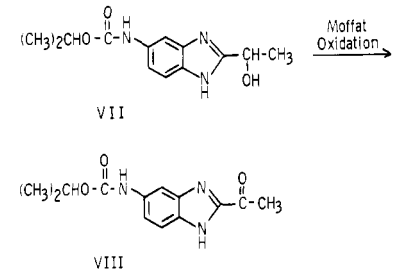


(<3%) of the m/e 265 isotope peak of the molecular ion indicated the absence of sulfur, and this was confirmed by high-resolution MS, empirical formula of $C_{13}H_{17}N_3O_3$. The metabolite formed a $(Me_3Si)_3$ derivative (molecular ion m/e 479) with M − 42 and M − 132 ions confirming the presence of the isopropoxycarbonylamino side chain. Room temperature acetylation (pyridine) resulted in a shift in molecular ion to m/e 347, indicating two readily acylable groups. The thiazole ring was clearly the site of metabolic change, and possible structures for metabolite VII were the isomeric hydroxyethyl alcohols. Compounds VII and XIV were both available for comparison with the metabolite, and the secondary alcohol exhibited exact correspondence by MS and TLC. The two isomeric alcohols were synthesized as outlined in Scheme VI.

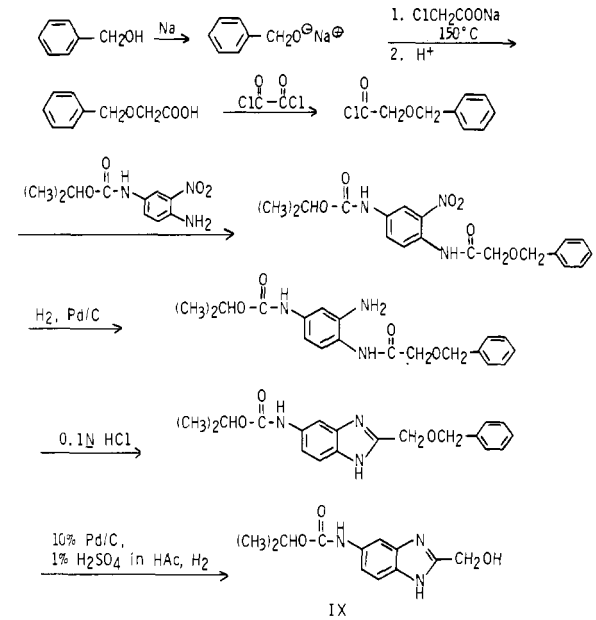
Metabolite VIII. XAD-2 chromatography of ovine urine gave a radioactive component designated metabolite VIII. The XAD-2 eluate fraction was partitioned between methylene chloride and water. The methylene chloride soluble fraction was subjected to MS examination after TLC purification on silica gel (solvent system I, R_f 0.8; system II, R_f 0.9).

A molecular ion of m/e 261 was observed using the direct probe MS technique, with fragment ions at M − 42 (m/e 219) and M − 60 (m/e 201). Following reaction with methoxyamine·HCl, the molecular ion increased by 29 amu to m/e 290, indicating that the metabolite contains an aldehyde or keto group. Gas–liquid radiochromatography of the fraction indicated that most of the eluted radioactivity was associated with one component. Combined GLC–MS of the component gave a "cleaner" spectrum than that obtained by direct probe, but with the same molecular ion and fragmentation pattern. As the secondary alcohol VII had previously been identified as a metabolite of CBZ, the corresponding ketone was thought

Scheme VII



Scheme VIII

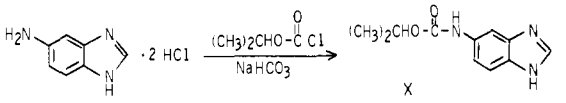


to be a likely candidate for the new metabolite. The ketone with structure VIII was prepared and found to possess the same GLC–MS and TLC properties as the metabolite. Further, the *O*-methyloxime of the ketone yielded a mass spectrum identical with that of the similarly derivatized metabolite. Metabolite VIII was synthesized by oxidation of metabolite VII as indicated in Scheme VII.

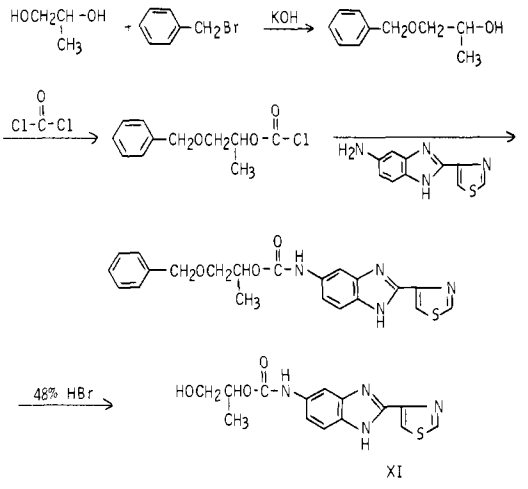
Metabolite IX. Metabolite IX was isolated by ethyl acetate extraction of bovine urine and purified by TLC on silica gel. When developed with solvent systems I–III, R_f values of 0.45, 0.7, and 0.5 were found. A mass spectrum obtained on this urine isolate indicated a probable molecular weight of 249; an intense fragment ion was observed at m/e 207, M − 42. Room temperature trimethylsilylation indicated the presence of one readily derivatized functional group (shift of molecular ion to m/e 321); derivatization at 50 °C gave low intensity signals of higher m/e values, suggesting the presence of two less reactive functional groups (shift in molecular ion to 465). Structure VIII was proposed, and authentic hydroxymethyl compound was prepared and found to be identical (MS, TLC) to the metabolite. Its synthesis was accomplished by the reactions outlined in Scheme VIII.

Metabolite X. This metabolite was found in the ethyl acetate extractable fraction of bovine urine. TLC on silica gel developed with benzene–ethyl acetate (80:20) gave an R_f of 0.5; with solvent systems I and II R_f values of 0.45 and 0.6 were found. Metabolite X exhibited $\lambda_{max}^{CH_3OH}$ 289 of $E^{1\%,1\ cm}$ 298. The infrared spectrum indicated carbonyl absorption at 1690 cm^{-1} (isopropoxycarbonylamino) and NH bending at 1530 cm^{-1}. Direct probe MS indicated an apparent molecular ion of m/e 219, with intense fragment ions at M − 42 and M − 60. The intensity of the M + 2

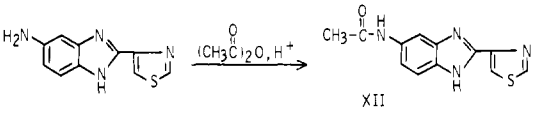
Scheme IX



Scheme X



Scheme XI



isotope peak at m/e 221 was too low for a compound containing sulfur. On the basis of these data 5-isopropoxycarbonylaminobenzimidazole was synthesized and found to possess the same MS and TLC properties as the metabolite. Its synthesis from 5-aminobenzimidazole was as shown in Scheme IX.

Metabolite XI. A metabolite of CBZ isolated by ethyl acetate extraction of porcine urine was purified by TLC on silica gel using solvent systems I and II (R_f values of 0.5 and 0.6, respectively). It possessed a molecular weight (direct probe MS) of 318, 16 units greater than the molecular weight of CBZ, suggesting that the metabolism involves insertion of an oxygen atom. The metabolite exhibited no M - 42 ion, but rather M - 58 (42 + 16), m/e 260. The next lower fragment observed is m/e 242 [M - (58 + 18)], the same m/e value as found with CBZ. These data suggest that the metabolite and CBZ differ only in the isopropoxycarbonylamino side chain—the presence of an oxygen atom on the isopropyl group. This is supported by MS of the Me_3Si derivative of the metabolite, which shows no M - 42 [in contrast to CBZ $(Me_3Si)_2$], but rather M - 130 (M - $C_3H_5OMe_3Si$); M - 220 is also observed, rather than M - 132.

GLC–MS (240 °C) of the metabolite and its Me_3Si derivative gave spectra identical with those found from the analogous experiments with CBZ; thermal degradation to the isocyanate eliminates the structural difference between the metabolite and the parent drug. High-resolution MS on the ion of m/e 318 proved that the metabolite is "oxy-CBZ", $C_{14}H_{14}O_3N_4S$. A sample of racemic XI was prepared (see Scheme X) and found to possess GLC, MS, and TLC properties identical with those of the metabolite.

Metabolite XII. Metabolite XII was found in the ethyl acetate extractable fraction from porcine urine and purified by TLC on silica gel developed with system II (R_f 0.5). MS of this metabolite indicated a molecular ion of m/e 258, with intense fragment ions at M - 42 and M - (42 + 27). The intensity of the M + 2 isotope peak suggested the presence of a sulfur atom in the molecule. The loss of 27 mass units (HCN?) suggested that the thiazole ring was still intact, in line with the presence of a sulfur atom. Mass spectrometry of the trimethylsilylated metabolite indicated the presence of two active hydrogen atoms (molecular ion of 402; 258 + 72 + 72).

The mass spectrum of the Me_3Si derivative obtained via combined GLC (240 °C)–MS was the same as that obtained via the direct probe system. As Me_3Si derivatives of compounds with the isopropoxycarbonylamino side chain (e.g., CBZ) are known to undergo thermal transformation to the corresponding isocyanate (O=C=N-R) when subjected to GLC at >200 °C, the substituent at the 5-position is clearly not that in cambendazole. High-resolution MS indicated a molecular formula of $C_{12}H_{10}ON_4S$. A sample of authentic 5-*N*-acetylamino-TBZ was prepared as shown in Scheme XI and found to possess a mass spectrum identical with that of the metabolite. The reference compound also exhibited the same R_f in TLC as the metabolite, and the GLC–MS behavior of the trimethylsilylated 5-*N*-acetylamino-TBZ was identical with that of the similarly derivatized metabolite.

Metabolite XIII. Separation experiments had revealed the presence in urine from CBZ-treated animals (sheep and pig) of a metabolite that turned pink and then buff-colored after exposure to air and light on TLC plates (solvent system I, R_f 0.35; solvent system II, Rf 0.5). A test for phenols (coupling with diazotized sulfanilic acid) was positive; the test for an aromatic amine (Bratton-Marshall) was negative, suggesting a ring-hydroxylated, i.e., phenolic, metabolite. Inspection of the ethyl acetate–water eluate from XAD-2 resin by TLC showed a fraction especially rich in this "pink spot" material. The fraction was evaporated to dryness and partitioned between methylene chloride and water; the water phase was then extracted with ethyl acetate. The ethyl acetate extract was examined by direct probe MS and TLC.

The apparent molecular weight of the metabolite, as determined by direct probe MS, was 274 (base peak) and on the basis of the "M + 2" isotope peak intensity (6%) XIII contained one S atom. The mass spectrum showed an intense fragment ion at M - 42, m/e 232, and also an ion at M - (42 + 27) (m/e 205). The mass spectrum of metabolite XIII is similar to that of 5-*N*-acetylamino-TBZ, except that the signals [M, M - 42, M - (42 + 27)] are shifted upward in m/e value by 16 units. When subjected to acetylation conditions a new molecular ion was observed at m/e 358, indicating the presence of two acylable groups. Trimethylsilylation increased the molecular weight of the compound from 274 to 490, suggesting the presence of three active hydrogen-containing groups (when BSA-d_{18} was employed a molecular ion of m/e 517 was observed). For comparison, 5-*N*-acetylamino-TBZ forms a $(Me_3Si)_2$ derivative, molecular ion of m/e 402. Under mild trimethylsilylation conditions (BSA, 30 °C, 1 min) which would be expected to form a Me_3Si derivative with only very reactive hydroxyl groups, the metabolite is transformed to a Me_3Si derivative. These observations are consistent with structure XIII.

Metabolite XIV. The finding of metabolite X suggested that its parent amine, 5-aminobenzimidazole (XIV), might also be present in urine. Reverse isotope dilution analysis (Walker et al., 1972) for XIV demonstrated the presence in ovine urine of 5-aminobenzimidazole (~1% of the total urinary radioactivity).

DISCUSSION

The large majority of identified urinary metabolites of CBZ result from structural transformations of the thiazole

ring. It is clear that several subgroups of thiazole-degraded metabolites exist, including (a) that in which the C-2 thiazole atom remains bonded to the nitrogen atom (II and III), (b) that in which the C-2 thiazole atom remains bonded to sulfur (I), and (c) that in which the metabolites possess a two-carbon side chain (VI, VII, VIII). Metabolite V could arise from IX by oxidation or IV by hydrolysis. The source of metabolite X is undoubtedly V via decarboxylation.

Metabolites I–X all possess the intact side chain at the 5-position, and those metabolites exhibiting structural change involving the isopropoxycarbonylamino moiety (XI–XIII) possess intact thiazole rings. Hydroxylation of the alkyl portion of the C-5 side chain is not unexpected as such transformations have been observed with parbendazole (Dunn et al., 1973), and extensive metabolism of a heterocyclic ring substituent on the 2-position of a benzimidazole has been reported (Frank, 1971), namely, the conversion of 2-(2-furyl)benzimidazole to 4-(2-benzimidazolyl)-4-hydroxybutyric acid. The transamidation leading to metabolite XII presumably involves hydrolysis of CBZ to 5-amino-TBZ followed by acetylation, but no evidence has been found for the excretion of this amine. The finding of 5-aminobenzimidazole demonstrates that metabolic changes involving the thiazole ring and the isopropoxycarbonylamino side chain are not mutually exclusive. XIII is the only other known metabolite which displays metabolic transformation of CBZ at more than one site. Parbendazole has been found to yield a phenolic metabolite (hydroxyl group ortho to the butyl side chain) (Dunn et al., 1973). It is a possibility, of course, that species in addition to XIII and XIV with metabolic changes involving several parts of the CBZ molecule were not isolated and characterized.

The array of urinary metabolites arising from CBZ when compared to the relatively simple metabolism picture reported for TBZ further demonstrates that with benzimidazole derivatives in which the 5-position is substituted, metabolic switching occurs to bring about oxidations by a variety of alternate pathways.

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