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Potential Antitumor Agents *via* Inhibitors of L-Asparagine Synthetase: Substituted Sulfonamides and Sulfonyl Hydrazides Related to Glutamine

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Received November 21, 1977, from the **Organic Chemistry Division, Microbiological Associates, Bethesda, MD 20016*, and the ‡*Laboratory of Toxicology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.* Accepted for publication March 7, 1978. §Present address: Food and Drug Administration, HFF-144, DCH/RAMIB, Washington, DC 20204.

Abstract □ A series of 4-(substituted aminosulfonyl)- and 4-(substituted hydrazinosulfonyl)-2-aminobutanoic acids, compounds structurally related to glutamine, was synthesized as potential inhibitors of L-asparagine synthetase and subjected to screening as antitumor agents. Target amino acids were obtained by condensation of a blocked reactive sulfonyl chloride with the appropriate amine or hydrazide, followed by deblocking with hydrogen–palladium or liquid hydrogen fluoride–anisole. Neither the target compounds nor their protected precursors inhibited the enzyme from L5178Y/AR or prolonged the life of mice with P-388 lymphocytic leukemia. However, DL-4,4′-dithiobis[2-(benzyloxycarbonylamino)butanoic acid], an intermediate in the synthesis of the target amino acids, exhibited 90% inhibition of L-asparagine synthetase at 10 m*M*.

Keyphrases □ Sulfonamides, various—synthesized, evaluated for antineoplastic activity in mice and effect on L-asparagine synthetase activity *in vitro* □ Sulfonyl hydrazides, various—synthesized, evaluated for antineoplastic activity in mice and effect on L-asparagine synthetase activity *in vitro* □ Antineoplastic activity—various sulfonamides and sulfonyl hydrazides evaluated in mice □ L-Asparagine synthetase activity—effect of various sulfonamides and sulfonyl hydrazides evaluated *in vitro* □ Enzyme activity—effect of various sulfonamides and sulfonyl hydrazides on L-asparagine synthetase evaluated *in vitro* □ Structure–activity relationships—various sulfonamides and sulfonyl hydrazides evaluated for antineoplastic activity in mice and effect on L-asparagine synthetase activity *in vitro*

Asparaginase, the enzyme responsible for hydrolyzing L-asparagine to L-aspartic acid, is therapeutically useful in the treatment of certain tumors (1). However, its effectiveness is diminished by several factors, including an induced resistance by formerly susceptible tumor cells. Various reports (2–4) indicated that such resistance is attributable to the appearance of high levels of the biosynthetic enzyme L-asparagine synthetase (I), which catalyzes the transfer of the amide nitrogen of L-glutamine to L-aspartic acid to form L-asparagine (5). The relation between resistance to asparaginase and high levels of I and sensitivity to asparaginase and absence of I provides a distinct biochemical difference between certain tumors (high levels of I) and normal tissue (low levels of I), which should be capable of being exploited chemotherapeutically.

In studies aimed at developing inhibitors of I and potential antitumor agents, Brynes *et al.* (6) recently described the synthesis and biological activity of asparagine analogs characterized by replacement of the amide carbonyl with a sulfonyl group. The compounds generally produced moderate (30–40% at 2 m*M*) inhibition of I isolated from Novikoff hepatoma, although 3-(hydroxysulfamoyl)-L-alanine displayed quantitative inhibition at 2 m*M* (6). Noting the importance of glutamine as a substrate for I (as well as for other significant biochemical events such as the biosynthesis of purines), this report describes the preparation and biochemical testing of several similar sulfonamides and sulfonyl hydrazides (VI*a*–VI*g*, Scheme I).

EXPERIMENTAL[1]

Chemistry—The route employed in obtaining target compounds VI*a*–VI*g* was analogous to that established previously (6) for a series of L-asparagine analogs. Generally, homocystine was fully protected by treatment first with benzyloxycarbonyl chloride and then with benzyl bromide in the presence of dicyclohexylamine. Subsequent cleavage of the blocked homocystine (III) with chlorine in aqueous acetic acid provided the reactive sulfonyl chloride IV, which was, in turn, allowed to react with the appropriate amine or hydrazide, yielding the blocked intermediates V*a*–V*g*. Hydrogenolysis or treatment with hydrogen fluoride gave the desired free amino acids (Scheme I). All compounds were characterized by their melting points, elemental analyses, IR spectra, and homogeneity on TLC (visualization with ninhydrin).

DL-4,4′-Dithiobis[2-(benzyloxycarbonylamino)butanoic Acid] (II)—This protected amino acid was prepared from DL-homocystine and benzyloxycarbonyl chloride in exactly the same manner as described for *N,N′*-di(benzyloxycarbonyl)-L-cystine (7). The use of 5.3 g (0.02 mole) of homocystine afforded 9.2 g (86%) of crystalline product from chloroform, mp 119–127°. Recrystallization from ethanol–water yielded an analytical sample, mp 138–139.5°.

Anal.—Calc. for $C_{24}H_{28}N_2O_8S_2$: C, 53.72; H, 5.26; N, 5.22. Found: C, 53.90; H, 5.44; N, 5.36.

Dibenzyl DL-4,4′-Dithiobis[2-(benzyloxycarbonylamino)butanoate] (III)—Diacid II (26.8 g, 0.05 mole), benzyl bromide (13.6 ml, 0.10 mole), and dicyclohexylamine (21.6 ml, 0.11 mole) were combined in 500 ml of dimethylformamide and allowed to stir overnight at room temperature. Following removal of the solvent *in vacuo*, the white solid was thoroughly

[1] Melting points were determined on a Mel-Temp apparatus and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. The petroleum ether used had a boiling-point range of 30–60°. Commercially unavailable monoethyl fumarylhydrazide, needed for the synthesis of V*g*, was prepared as previously described (6).

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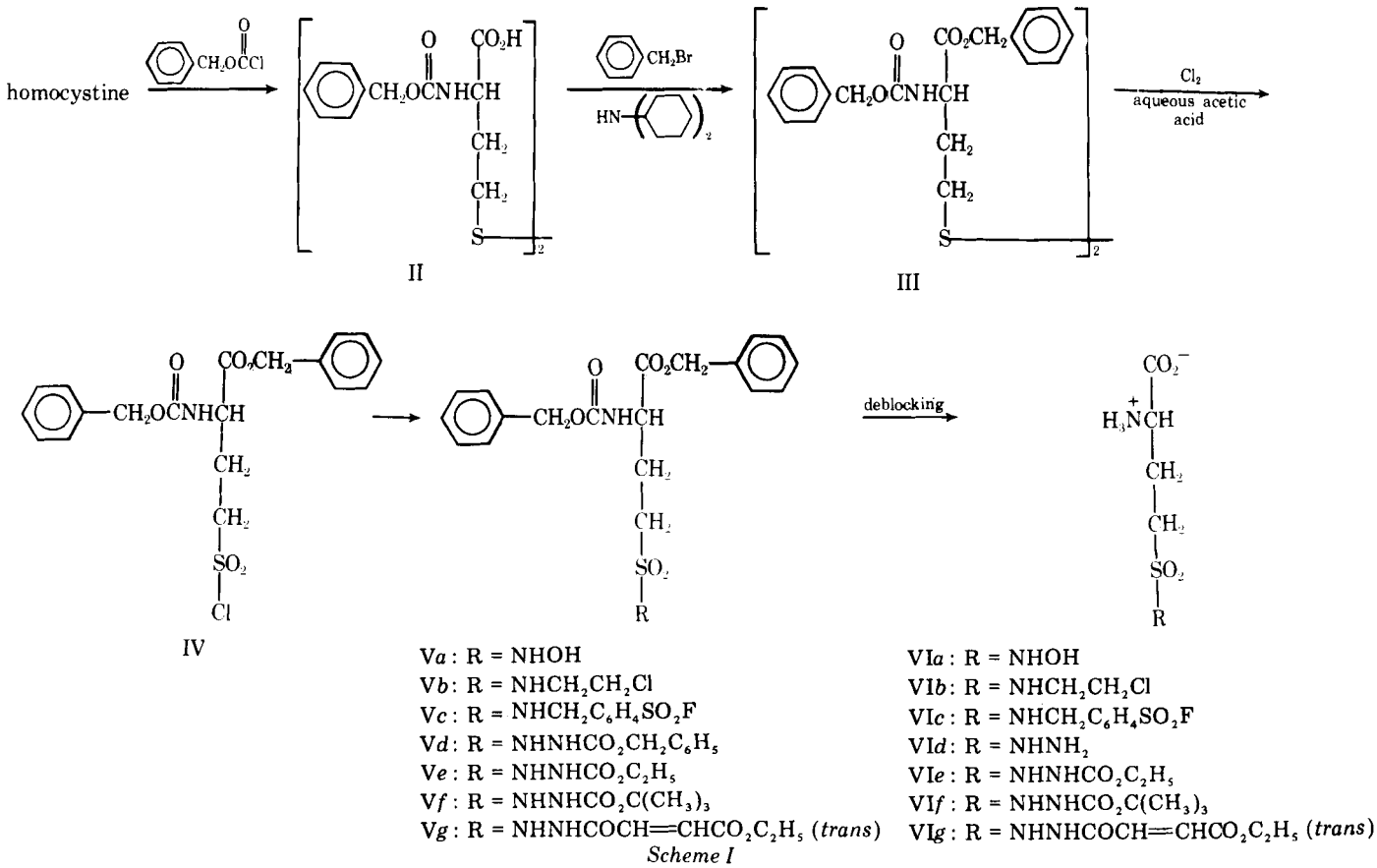

Scheme I

triturated with ethyl acetate. The insoluble material was removed by filtration, and the filtrate was washed twice with 1 *N* HCl, twice with 6.9% $NaHCO_3$, and once with 35% NaCl. The organic solvent was dried and evaporated. The oil remaining was crystallized from ether–petroleum ether, affording 28.9 g (81%) of desired product III, mp 84–101°. Repeated recrystallization from the same solvent system yielded an analytical sample, mp 92–96°.

Anal.—Calc. for $C_{38}H_{40}N_2O_8S_2$: C, 63.67; H, 5.62; N, 3.91. Found: C, 63.66; H, 5.72; N, 3.99.

Benzyl DL-4-(Chlorosulfonyl)-2-(benzyloxycarbonylamino)butanoate (IV)—Diester III (21.5 g, 0.03 mole) was dissolved in 700 ml of acetic acid containing 2 ml of water. Chlorine (14 ml, 0.30 mole) was condensed in a test tube surrounded by dry ice–acetone. When the tube was removed from the ice bath, the chlorine slowly distilled and was introduced just above the surface of the reaction solution, which was stirred at 0°. Following complete distillation of the chlorine, the reaction mixture was allowed to warm to room temperature, and solvent was removed by co-distillation with carbon tetrachloride. The remaining oil was crystallized from ether–petroleum ether, yielding 18.8 g (70%) of sulfonyl chloride IV, mp 51–57° (softens) and 57–61° (melts). Preparative TLC (silica, chloroform) afforded an analytical sample, mp 39–42°.

Anal.—Calc. for $C_{19}H_{20}ClNO_6S$: C, 53.58; H, 4.73; N, 3.29. Found: C, 53.66; H, 4.76; N, 3.45.

Protected 4-(Substituted aminosulfonyl)- and 4-(Substituted hy-

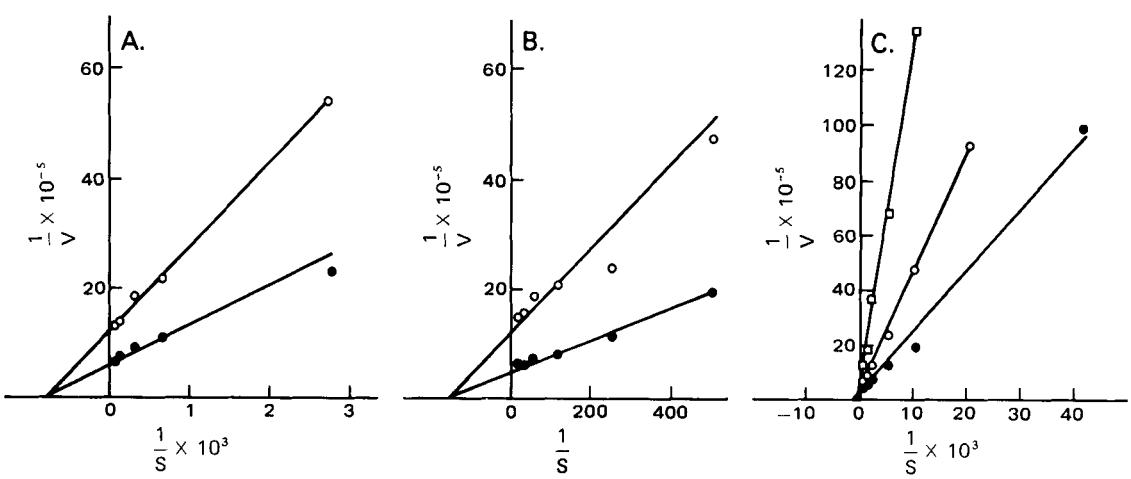

Figure 1—*Double-reciprocal plots of II* versus *L-glutamine, ammonium chloride, and L-aspartic acid for asparagine synthetase of L5178Y/AR. In a final volume of 15 μl were admixed 4 (○) or 8 (□) mM II or water (●), ATP (10 mM), magnesium chloride (25 mM), and the following: A, L-glutamine (13, 6, 3, 1.5, or 0.35 mM) and L-4-^{14}C-aspartic acid (0.05 mM); B, ammonium chloride (66, 33, 17, 8, 4, or 2 mM) and L-4-^{14}C-aspartic acid (0.056 mM); and C, L-4-^{14}C-aspartic acid (specific radioactivity of 12.9 μCi/μmole; 1.46, 0.74, 0.37, 0.18, 0.09, 0.045, and 0.02 mM) and L-glutamine (13 mM). A 5-μl aliquot of the 27,000×g (20 min) supernate of L5178Y/AR homogenate (1:4, w/v) was used to initiate the reaction. All vessels were incubated at 37° for 30 min, after which the reaction was terminated by heating the closed vessels at 95° for 5 min. Subsequent steps for the recovery of synthesized L-4-^{14}C-asparagine were conducted as previously described (9). V = cpm; S = molarity.*

Table I—4-(Substituted aminosulfonyl)- and 4-(Substituted hydrazinosulfonyl)-2-aminobutanoic Acids and Antitumor Activity [a]

Compound	Reaction Solvent (Method) [b]	Yield, %	Melting Point	Recrystallization Solvent	Formula		Analysis, % Calc.	Analysis, % Found	Antitumor Activity Dose [c]	Antitumor Activity T/C [d]
II	*Experimental* section	86	138–139.5°	Ethanol–water	$C_{24}H_{28}N_2O_8S_2$	C	53.72	53.90	400 [e]	103
						H	5.26	5.44		
						N	5.22	5.36		
III	*Experimental* section	81	92–96°	Ether–petroleum ether	$C_{38}H_{40}N_2O_8S_2$	C	63.67	63.66	400 [e]	94
						H	5.62	5.72		
						N	3.91	3.99		
V*a*	1% Water–acetonitrile	30	112–114°	Ether–petroleum ether	$C_{19}H_{22}N_2O_7S$	C	54.02	53.86	400 [e]	109
						H	5.25	5.37		
						N	6.63	6.43		
V*b*	Chloroform–water [f]	34	91–93°	Carbon tetrachloride	$C_{21}H_{25}ClN_2O_6S$	C	53.79	53.57	400 [e]	92
						H	5.37	5.15		
						N	5.97	5.80		
V*c*	Acetonitrile	55	114–115°	Ethyl acetate–petroleum ether	$C_{26}H_{27}FN_2O_8S_2$	C	53.97	54.06	200 [e]	98
						H	4.70	4.80		
						N	4.84	4.76		
V*d*	Chloroform	54	123°	Carbon tetrachloride	$C_{27}H_{29}N_3O_8S$	C	58.37	58.13	400 [e]	111
						H	5.26	5.10		
						N	7.56	7.44		
V*e*	Chloroform	59	114–118°	Ethanol–water	$C_{22}H_{27}N_3O_8S$	C	53.54	53.53	400 [e]	91
						H	5.51	5.65		
						N	8.51	8.54		
V*f*	Chloroform	76	68°	Carbon tetrachloride	$C_{24}H_{31}N_3O_8S$	C	55.27	55.12	400 [e]	93
						H	5.99	6.11		
						N	8.06	7.97		
V*g*	Pyridine	25	143–145°	Ethyl acetate–petroleum ether	$C_{25}H_{29}N_3O_9S$	C	54.84	54.74	200 [e]	86
						H	5.34	5.40		
						N	7.67	7.66		
VI*a*	A	86	186–187° dec.	Water–ethanol	$C_4H_{10}N_2O_5S$	C	24.24	24.33	400 [g]	101
						H	5.09	5.16		
						N	14.13	14.15		
VI*b*	A	82	230° dec.	Water	$C_6H_{13}ClN_2O_4S$	C	29.45	29.59	400 [g]	99
						H	5.36	5.36		
						N	11.45	11.29		
VI*c*	B	58	226–230°	Water	$C_{11}H_{15}FN_2O_6S_2$	C	37.28	37.51	200 [g]	88
						H	4.27	4.42		
						N	7.90	7.96		
VI*d*	A	79	187–193° dec.	Water–ethanol	$C_4H_{11}N_3O_4S$	C	24.36	24.45	100 [g]	98
						H	5.62	5.75		
						N	21.31	21.26		
VI*e*	A	83	234–237°	Water	$C_7H_{15}N_3O_6S$	C	31.22	31.03	400 [g]	111
						H	5.61	5.70		
						N	15.60	15.51		
VI*f*	A	72	189° dec.	Water–acetone	$C_9H_{19}N_3O_6S$	C	36.36	36.18	400 [e]	93
						H	6.44	6.44		
						N	14.13	13.98		
VI*g*	B	58	203–204° dec.	Water–acetone	$C_{10}H_{17}N_3O_7S \cdot H_2O$	C	35.18	35.34	100 [g]	116
						H	5.61	5.73		
						N	12.31	12.45		

[a] Antitumor activity determined *versus* P-388 lymphocytic leukemia; QD 1–9 treatments, drug given ip. Protocols and tumor systems were described in Ref. 10. [b] The letter refers to general procedure given in *Experimental* section. [c] In milligrams per kilogram. [d] T/C (treated survival/control survival) × 100. [e] Water + Tween 80 as vehicle. [f] A two-phase solvent system was used in this synthesis: blocked sulfonyl chloride IV in chloroform and 2-chloroethylamine hydrochloride and sodium carbonate in an equal volume of water were combined with vigorous stirring. [g] Water as vehicle.

drazinosulfonyl)-2-aminobutanoic Acids (V*a*–V*g*)—For benzyl DL-4-(hydroxyaminosulfonyl)-2-(benzyloxycarbonylamino)butanoate (V*a*), sulfonyl chloride IV (20.0 g, 0.047 mole) in 1% aqueous acetonitrile was allowed to stir with hydroxylamine hydrochloride (13.0 g, 0.19 mole) and sodium bicarbonate (19.7 g, 0.24 mole) for 3 hr at room temperature. The solvent was removed *in vacuo*, and the remaining salts were washed with ethyl acetate. Following removal of the salts by filtration, the ethyl acetate was washed twice with 1 *N* HCl, 6.9% $NaHCO_3$, and 35% NaCl and dried. Removal of the ethyl acetate yielded an oil. This oil was purified with column chromatography (silica, 4% methanol–chloroform) and subsequently crystallized from ether–petroleum ether, affording 5.9 g (30%) of V*a*, mp 112–114°.

Anal.—Calc. for $C_{19}H_{22}N_2O_7S$: C, 54.02; H, 5.25; N, 6.63. Found: C, 53.86; H, 5.37; N, 6.43.

Compounds V*b*–V*g* were prepared similarly in the solvents indicated in Table I with reaction times varying between 3 and 6 hr. Triethylamine served as the acid acceptor for *p*-(fluorosulfonyl)benzylamine hydrochloride (8) in the synthesis of V*c*.

Preparation of Free Amino Acids VI*a*–VI*g*—*Method A*—Blocked amino acid (0.003 mole) was dissolved in 50 ml of 20% aqueous ethanol and hydrogenated at atmospheric pressure in the presence of 350 mg of palladium black. Following removal of the catalyst by filtration and evaporation of the filtrate, the residue was crystallized from the appropriate solvent (Table I). Yields were in the 70–90% range.

Method B—Blocked amino acid (0.01 mole) and anisole (0.02 mole) were allowed to stir with 20 ml of liquid hydrogen fluoride at 0° for 1 hr. Following complete removal of hydrogen fluoride by aspiration, the residue was dissolved in 100 ml of 6% aqueous acetic acid and washed several times with ethyl acetate. The aqueous solution was lyophilized, and the residue was crystallized from the appropriate solvent (Table I).

Preparation of I, Assay for I, and Inhibition—Generally, I was extracted from subcutaneous nodules of leukemia 5178Y resistant to L-asparaginase (L5178Y/AR) grown in BDF_1 mice. The 105,000×*g* supernate (specific activity of 0.001 IU/mg of protein) was used as the enzyme source. L-4-^{14}C-Aspartic acid was incubated with ATP, magnesium chloride, L-glutamine, and I; any L-4-^{14}C-asparagine synthesized was determined as previously described (9). Further details can be found in Ref. 9.

Antitumor Activity—Antitumor activity *versus* lymphocytic leukemia P-388 was determined by standard protocols[2] (10). Compounds were considered active if they gave T/C activity (10) values in the P-388 system equal to or greater than 125%, where T/C represents the ratio of

[2] Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.

the median survival times of the treated animals over those of the control animals expressed as a percentage.

RESULTS AND DISCUSSION

All synthesized compounds except IV were subjected both to an *in vitro* inhibition assay with I and to screening for antitumor activity. At 10 m*M*, II and VI*a* with glutamine as the nitrogen donor inhibited I by 90 and 32%, respectively; at 1 m*M*, both compounds were devoid of activity. The remainder of the compounds possessed negligible or no activity when tested at 1 and 10 m*M*. The results for the antitumor screen are given in Table I; none of the analogs displayed significant (T/C ≥ 125%) activity.

Only diacid II exhibited significant inhibition of I *in vitro*. In double-reciprocal plots (Fig. 1), II was competitive with L-aspartic acid for crude I, but noncompetitive with L-glutamine. From the results for II, one might expect that either homocystine or diester III would also show some inhibition with I; however, neither compound showed activity in the assay. Dithiothreitol (10 m*M*) did not prevent the inhibition of I by II, indicating that the agent was not acting by virtue of sulfhydryl groups. Since enzymes utilizing amino acids are usually highly substrate specific (11), it appears improbable that structurally bulky (as compared to aspartic acid) II is acting directly at the aspartic acid site of I. The observed inhibition probably results from an allosteric effect by II upon the aspartic acid site of the synthetase.

It is somewhat surprising that none of the target free amino acids of this study exhibited significant inhibitory action with I. In particular, results from an earlier study indicated 3-(hydroxysulfamoyl)-L-alanine (normethylene analog of VI*a*) to be a moderately good inhibitor of I from L5178Y/AR (81 and 23% at 10 and 1 m*M*, respectively) (6). Compound VI*a*, on the other hand, exhibited reduced activity (32 and 0% at 10 and 1 m*M*, respectively). Kinetic studies with 3-(hydroxysulfamoyl)-L-alanine and I revealed that the amino acid was acting as an analog of aspartic acid rather than of asparagine (6). By extrapolation, VI*a* becomes an analog not of glutamine but of glutamic acid. Because I does not utilize glutamic acid, the lowered activity of VI*a* is understandable.

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Radioimmunoassay of Oxfendazole in Bovine, Equine, or Canine Plasma or Serum

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Received October 31, 1977, from the *Department of Drug Metabolism, Syntex Research, Palo Alto, CA 94304.* Accepted for publication March 8, 1978.

Abstract □ A simple radioimmunoassay was developed for the determination of oxfendazole in plasma. Oxfendazole *N*-1(3)-valerate was coupled to polylysine *via* a carbodiimide reaction, and antiserum was developed in rabbits after inoculation with oxfendazole–polylysine conjugate. The assay was developed so that oxfendazole could be measured directly in a 0.1-ml aliquot of diluted or undiluted plasma. With the developed procedure, 200 pg of oxfendazole/ml of plasma can be determined quantitatively. Cross-reactivity was determined for closely related compounds and metabolites. The method was used to determine plasma concentration–time profiles in dogs and calves.

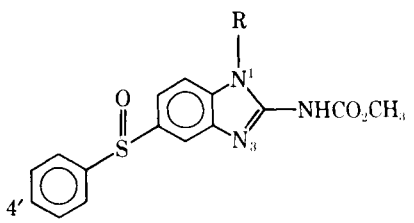
Keyphrases □ Oxfendazole—radioimmunoassay in plasma or serum □ Radioimmunoassay—oxfendazole in plasma or serum □ Anthelmintics—oxfendazole, radioimmunoassay in plasma or serum

Oxfendazole (I) is active against a broad spectrum of GI nematodes, lungworms, and tapeworms found in cattle, sheep, and pigs (1).

This paper describes an assay for I that was developed to provide data for the determination of certain pharmacokinetic parameters, *e.g.*, plasma clearance, half-lives, and absorption. The sensitivity requirement for these determinations was the low nanogram range.

EXPERIMENTAL

Materials—Freund's complete and incomplete adjuvants were purchased in sealed glass ampuls[1]. Activated powdered charcoal[2] was used.



I: R = H

II: R = $(CH_2)_4COOH$

[1] Difco Laboratories, Detroit, Mich.

[2] Norit A, Matheson, Coleman and Bell, Norwood, Ohio.

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