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

C. NERENBERG, R. A. RUNKEL, and S. B. MATIN ˣ

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.

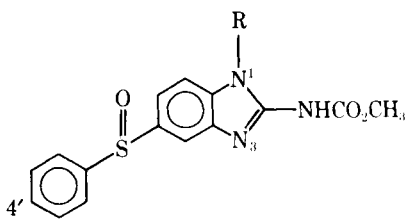
Keyphrases □ Oxfendazole—radioimmunoassay in plasma or serum □ Radioimmunoassay—oxfendazole in plasma or serum □ Anthelmintics—oxfendazole, radioimmunoassay in plasma or serum

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.

0022-3549/78/1100-1553$01.00/0

Table I—Radioimmunoassay Protocol

Tube Number	Description	Number of Replicates	Buffer, ml	Tritiated Oxfendazole, ml	Cold Oxfendazole, ml	Unknowns, ml	Antiserum, ml	Total Volume, ml
0	Zero standard	6	0.4	0.1	—	—	0.1	0.6
1	20-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
2	50-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
3	100-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
4	200-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
5	500-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
6	1000-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
7	1500-pg standard	3	0.3	0.1	0.1	—	0.1	0.6
N1[a], N2, *etc.*	Nonspecific traps (plasma)	6	0.4	0.1	—	0.1	—	0.6
NB[b]	Nonspecific trap (buffer)	3	0.5	0.1	—	—	—	0.6
TC	Total counts	3	0.5	0.1	—	—	—	0.6
U1, U2, *etc.*	Unknowns	3	0.3	0.1	—	0.1	0.1	0.6

[a] N1, N2, *etc.*, are nonspecific traps containing an aliquot of plasma equivalent to the dilutions used for the unknowns. [b] NB is the nonspecific trap for buffer only and it applies to the standard curve.

The buffer used throughout was 0.05 or 0.1 *M* phosphate-buffered saline, pH 8.0, with or without 0.1% gelatin.

Labeled oxfendazole was synthesized in-house[3] and was tritiated at the 4′-phenyl position with a specific activity of 24–27 Ci/mmole. The *N*-1(3)-valerate derivative (II) of oxfendazole and the oxfendazole–polylysine conjugate were synthesized in-house[3]. The oxfendazole derivatives used in the cross-reactivity experiments also were synthesized in-house[3].

Antiserum Production—The oxfendazole–polylysine conjugate used for immunization was prepared by coupling II to polylysine with a water-soluble carbodiimide coupling reagent (2). The reaction mixture was dialyzed exhaustively against normal saline. The extent of conjugation was determined by measuring the polylysine concentration by the method of Lowry *et al.* (3) and the oxfendazole residues by UV absorption. The molar ratio of oxfendazole to polylysine varied between 60 and 120 moles of oxfendazole/mole of polylysine.

An emulsion of the conjugate in saline was prepared by mixing with an equal volume of Freund's complete adjuvant. The polylysine concentration was 100 μg/ml, and 0.25 ml was injected subcutaneously into four sites in New Zealand White rabbits. Six weeks after the initial injection, all animals were placed on a regimen of weekly booster shots in which the conjugate was prepared in incomplete Freund's adjuvant. After 4–5 months, antiserum dilutions of 1:1000 and 1:2000 yielded 50% binding or more. At this stage, development of the assay was begun.

Radioimmunoassay—Oxfendazole standards for the standard curve were obtained by dilution of a stock solution of 1.0 mg of oxfendazole in 10.0 ml of propylene glycol–0.2 *N* HCl (1:1). The series of standard solutions, 20, 50, 100, 200, 500, 1000, and 1500 pg/0.1 ml, was prepared in 0.05 *M* phosphate-buffered saline, pH 8.0, without gelatin in 10.0-ml volumes and stored in the refrigerator. Gelatin was withheld from the buffer in the standards to give them a longer shelflife.

The labeled oxfendazole was prepared in 0.05 *M* phosphate-buffered saline, pH 8.0, with 0.1% gelatin such that a 0.1-ml aliquot contained approximately 10,000–12,000 cpm. This solution was normally prepared in a volume sufficient for one or two assays only and was not kept longer than 3–4 days. Radioisotope purity was checked periodically by TLC using chloroform–methanol (95:5).

The antiserum was diluted in 0.05 *M* phosphate-buffered saline, pH 8.0, with 0.1% gelatin to yield a total binding of between 35 and 50%. Depending on the quality of the antiserum, this binding was achieved by diluting 1:500, 1:1000, or 1:2000; a 0.1-ml aliquot was added to each tube.

The charcoal stock suspension (1% in water) was diluted as required with 0.05 *M* phosphate-buffered saline, pH 8.0, with 0.1% gelatin immediately before use. A 0.2-ml aliquot of the diluted charcoal suspension was added to blocks of 12–15 tubes at a time. The tubes were vortexed, and the 30-min time cycle was started. Midway through this cycle, additions were made to a second block of tubes and they were started on their 30-min cycle. This staggered array procedure was repeated until all tubes were processed. At the end of the 30-min cycle, each block of tubes was centrifuged immediately and sampled for counting.

A typical radioimmunoassay protocol is given in Table I, and a stepwise procedure for setting up the assay is as follows:

1. Add buffer, label, standards, unknown, antiserums, *etc.*, to the appropriate tubes. Use 12 × 75 disposable culture tubes.
2. After all additions have been made, vortex all tubes for 1–3 sec.
3. Cover the tubes with sheets of waxed film[4] cut to fit test tube racks.
4. Incubate the tubes overnight at 40° in a covered temperature-controlled water bath.
5. After overnight incubation, place the tubes in an ice bath and start the charcoal addition regimen. Add 0.2 ml of charcoal to blocks of 12–15 tubes. Vortex the tubes for 6 sec and then start the 30-min time cycle.
6. After 30 min, centrifuge the tubes at 2000 rpm for 4 min and immediately transfer 0.3 ml of the supernate into scintillation vials. Add 10.0 ml of scintillation fluid.
7. Count for 10 min.
8. Process the data with the Rodbard and Lewald Model I program (4) or manually plot percent bound *versus* oxfendazole concentration and determine unknowns by interpolation.

The centrifuge[5] used was a bench top model with swing out head. Liquid scintillation counting was done in a scintillation spectrometer[6], using commercially available scintillation fluid[7].

^{14}C-Radiochemical Assay—Three calves were given an oral dose of ^{14}C-oxfendazole (3 mg/kg) having the following specific activities: 8.8×10^6 dpm/mg for Calf 1 and 50.2×10^6 dpm/mg for Calves 2 and 3. Blood samples were taken at 2, 4, 8, 24, and 48 hr after dosing.

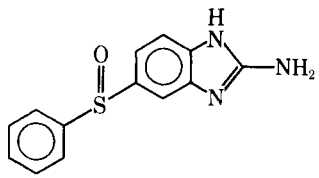
To 1.0 ml of plasma were added 0.5 ml of phosphate buffer, pH 8.0, and 2.0 ml of chloroform. The tubes were shaken and centrifuged, and the chloroform layer was removed. The extraction was carried out three times, and the chloroform extracts were combined. Then the chloroform extracts were taken to dryness under nitrogen, and the residue was resuspended in a minimum volume of solvent. The residue was spotted on TLC plates, and the peak corresponding to oxfendazole was scraped into a scintillation vial and counted in liquid scintillation fluid.

Specificity—Cross-reactivities were determined from displacement curves generated for oxfendazole free amine (III), oxfendazole thio ether (IV), oxfendazole free amine thio ether (V), and oxfendazole sulfone (VI). This family of curves was compared to the oxfendazole displacement curve. The concentration of each compound that corresponded to the 50% inhibition point on the oxfendazole curve was then used to calculate the percent of cross-reactivity.

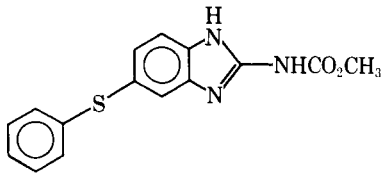
Data Processing, Sensitivity, and Precision—Data processing was done with the aid of the Rodbard and Lewald Model I computer program (4), a logit–log transformation that linearizes the sigmoid curve normally obtained in a displacement reaction. The advantages of this system are that it eliminates operator variation both in plotting the standard curve and estimating the unknowns and it increases the speed with which the computations can be made, especially if a teletype punched tape system is coupled to the program. Since the program does not have the capability to subtract different nonspecific traps, this step was either done manually

[3] Syntex Research, Palo Alto, Calif.

[4] Parafilm.
[5] IEC centrifuge model HN-S.
[6] Packard Tri-Carb model 3330.
[7] Aquasol or Oxifluor-H_2O, New England Nuclear.



III



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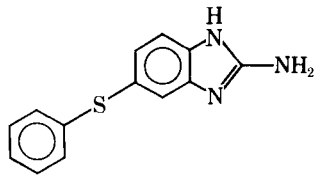

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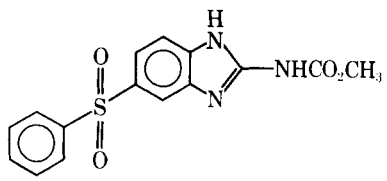

VI

beforehand or by the use of a small preprogram that then fed the corrected data into the Rodbard and Lewald program.

The accuracy and precision of the assay were determined by adding known amounts of oxfendazole to equine, bovine, and canine plasma or serum and then assaying six replicates of each concentration for each plasma.

RESULTS AND DISCUSSION

Assay Conditions—The parameters optimized in this assay were the charcoal contact time and its influence on the separation of bound from free oxfendazole, the effect of pH on total binding, the effect of temperature on the antigen–antibody equilibrium process, and the optimum amount of charcoal required for the separation of bound from free oxfendazole.

The amount of charcoal used to separate bound from free oxfendazole (5) was such that the buffer nonspecific bound would run from 2 to 4% of the total radioactivity added and the nonspecific bound containing an aliquot of undiluted plasma would range from 3 to 8%.

The charcoal contact time is defined as that interval from the instant of addition to when an aliquot is removed for counting. Figure 1 illustrates the time dependence of this process. The procedure outlined, *i.e.*, processing small blocks of tubes at a time, had as its primary purpose to maintain the contact time as nearly alike for all tubes as possible. It would be difficult to maintain a constant time for a large block of 200–300 tubes. However, by processing a staggered array of smaller blocks of tubes, this factor could be controlled.

The effect of buffer pH on the total binding obtained showed that slightly higher binding was achieved at pH 8.0 than at lower pH values. Therefore, pH 8.0 buffers were routinely employed.

The effect of incubation temperature on the antigen–antibody reaction was investigated, and the results failed to show any clearcut advantage. Assays were carried out at 40° in a constant-temperature water bath or at room temperature.

The final assay conditions chosen were as follows. The amount of charcoal required to maintain the nonspecific bound in the specified range was around 0.4%, the charcoal contact time was 30 min plus the time required for centrifugation and aliquoting, the buffer pH was 8.0, and the radioimmunoassay incubation was carried out overnight at 40° or room temperature.

Table II—Validity of Using Nonspecific Traps to Correct Serum or Plasma Unknowns

Experiment	Oxfendazole Added, ng/0.1 ml of Serum	Oxfendazole Measured, ng/0.1 ml of Serum	Ratio of Measured to Added
A[a]	0.50	0.52 ± 0.013[b]	1.04
	1.00	1.08 ± 0.014	1.08
	1.50	1.49 ± 0.037	0.99
	2.00	1.96 ± 0.054	0.98
B[c]	0.50	0.43 ± 0.015	0.86
	1.00	1.04 ± 0.017	1.04
	1.50	1.50 ± 0.038	1.00

[a] In Experiment A, the standard curve was run in buffer only. Samples were prepared by fortifying with known amounts of oxfendazole in the presence of a 0.1-ml aliquot of serum diluted 1:10. The nonspecific trap contained an equivalent amount of diluted serum. The samples were corrected by subtracting the nonspecific bound and then reading these values from the buffer standard curve. [b] Standard errors calculated from six replicates. [c] In Experiment B, the standard curve was run in buffer only. The fortified samples and nonspecific traps were prepared in undiluted plasma but otherwise were treated as in Experiment A.

In this assay, the appropriate nonspecific bound values were subtracted from the unknowns and these values were then interpolated from a

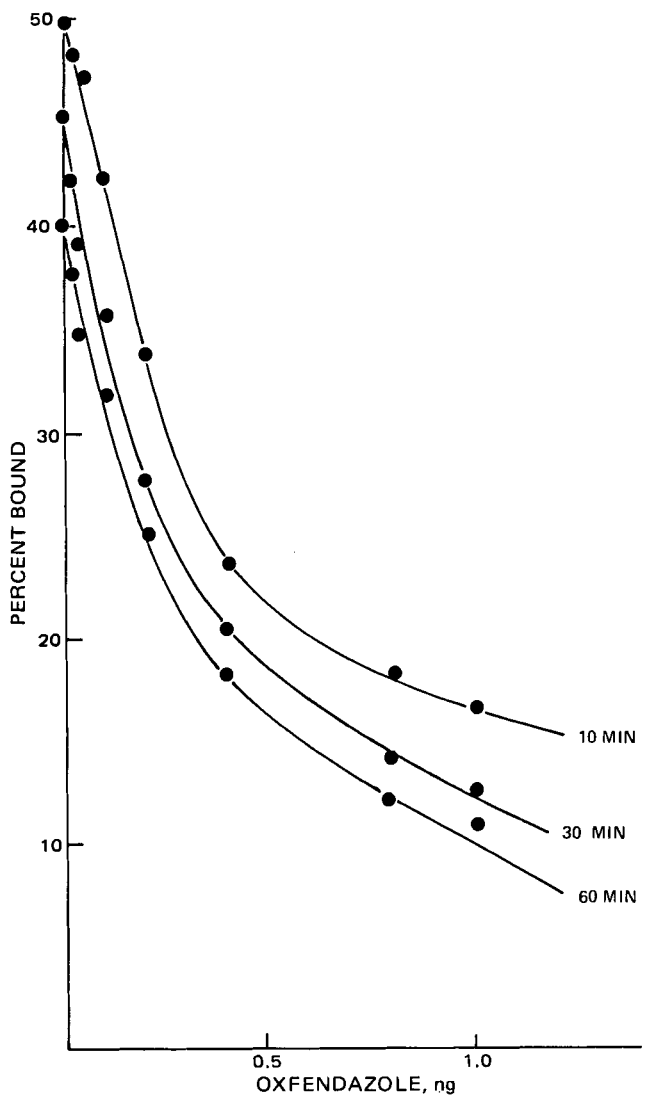

Figure 1—*Effect of charcoal contact time on the separation of bound from free radioactivity. Three displacement curves were run simultaneously under identical conditions while the charcoal contact time was varied.*

Table III—Estimation of Oxfendazole in Bovine Plasma

Oxfendazole Added, ng	Oxfendazole Measured, ng	Mean ± *SE*	Ratio of Assayed to Added
0.20	0.14	0.16 ± 0.006	0.80
	0.16		
	0.17		
	0.15		
	0.18		
	0.17		
0.50	0.53	0.52 ± 0.013	1.04
	0.49		
	0.51		
	0.53		
	0.58		
	0.50		
1.00	1.12	1.08 ± 0.014	1.08
	1.08		
	1.11		
	1.08		
	1.02		
	1.08		
1.50	1.40	1.49 ± 0.037	0.99
	1.52		
	1.44		
	1.45		
	1.66		
	1.49		
2.00	2.08	1.96 ± 0.054	0.98
	1.95		
	2.10		
	1.82		
	1.78		
	2.00		
2.50	2.39	2.52 ± 0.069	1.01
	2.33		
	2.80		
	2.56		
	2.44		
	2.60		

standard curve run in buffer only. The data in Table II are given in support of this procedure. The appropriate nonspecific bound simply means that if the plasma samples are run undiluted, then the nonspecific bound tubes also consist of an aliquot of undiluted plasma; if the unknowns are diluted, then there must be correspondingly diluted nonspecific bound tubes. The alternative would be to add an aliquot of untreated plasma to all points of the standard curve, thereby creating a chemical environment equivalent to the unknowns.

Specificity—To investigate cross-reactivities, a number of known oxfendazole metabolites (III–VI) were selected. The sulfide (IV) and

Table IV—Estimation of Oxfendazole in Equine Serum

Oxfendazole Added, ng	Oxfendazole Measured, ng	Mean ± *SE*	Ratio of Assayed to Added
0.200	0.198	0.210 ± 0.009	1.05
	0.206		
	0.184		
	0.228		
	0.238		
0.500	0.479	0.429 ± 0.021	0.86
	0.407		
	0.504		
	0.419		
	0.405		
	0.359		
1.00	1.02	1.02 ± 0.064	1.02
	0.91		
	0.78		
	1.09		
	1.19		
	1.17		
2.00	2.58	2.19 ± 0.085	1.09
	2.25		
	1.99		
	2.18		
	2.06		
	2.11		

Table V—Estimation of Oxfendazole in Canine Plasma

Oxfendazole Added, ng	Oxfendazole Measured, ng	Mean ± *SE*	Ratio of Assayed to Added
0.05	0.040	0.041 ± 0.0027	0.82
	0.045		
	0.030		
	0.043		
	0.038		
	0.050		
0.10	0.100	0.082 ± 0.0045	0.82
	0.070		
	0.075		
	0.090		
	0.080		
	0.077		
0.50	0.520	0.466 ± 0.014	0.94
	0.485		
	0.475		
	0.450		
	0.435		
	0.435		
1.0	1.20	1.05 ± 0.12	1.05
	0.84		
	1.60		
	0.88		
	0.85		
	0.97		

sulfone (VI) metabolites known to circulate in bovine and sheep plasma after oral oxfendazole cross reacted to the extent of 2.00 and 0.25%, respectively. Compound V cross reacted at 0.33%. The oxfendazole free amine (III) showed higher cross-reactivity, 11.3%. Although this metabolite was present in the urine of sheep and cattle, its presence was not established in plasma. Thus, the major cross-reacting metabolite very likely does not interfere in the plasma radioimmunoassay.

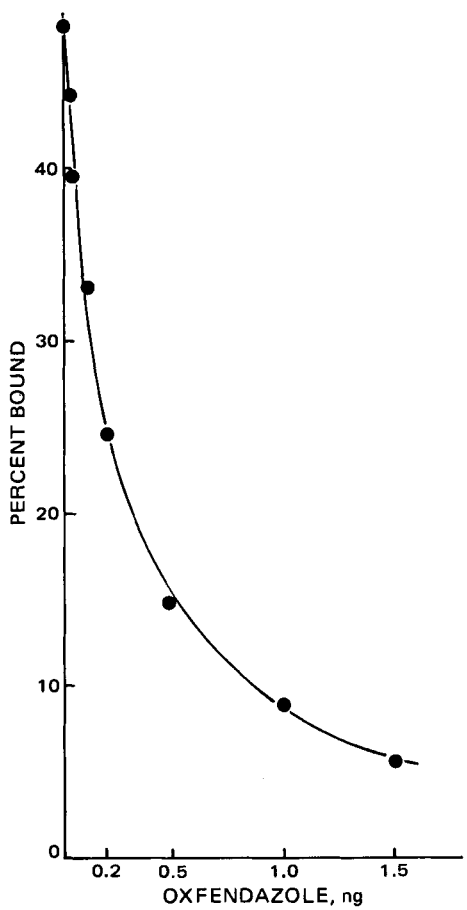

Figure 2—*Plot of the displacement curve (or standard curve). The antiserum dilution was 1:1000. The reaction was carried out overnight at room temperature.*

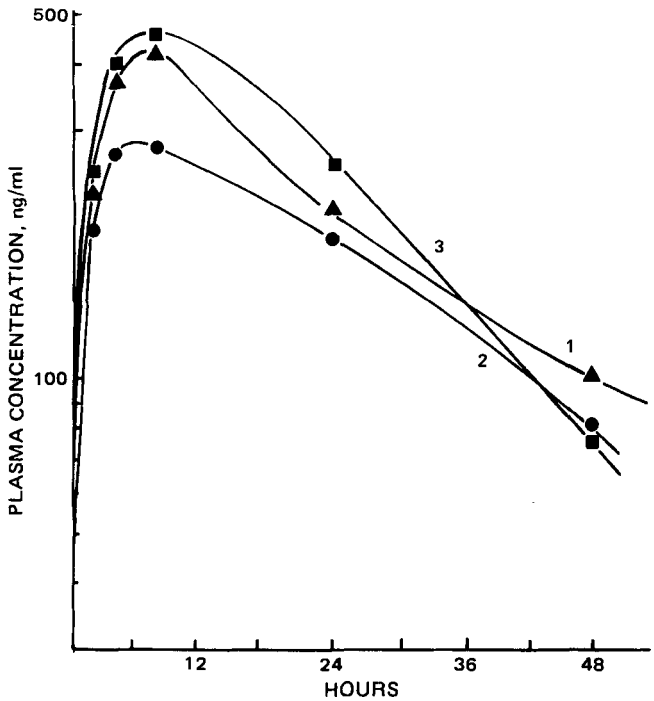

Figure 3—*Plot of the plasma concentration–time profile in calves after an oxfendazole dose of 3 mg/kg.*

Sensitivity, Accuracy, and Precision—A typical displacement (or standard) curve is shown in Fig. 2. The displacement curve is linear in logit–log coordinates in the range of 20–1500 pg. The displacement curve shows the 20-pg point to be uniquely different from zero, and it was taken as the lower limit of sensitivity. In this assay, this limit corresponds to a value of 200 pg/ml of plasma or serum.

Accuracy and precision of the assay were determined by adding known amounts of oxfendazole to plasma or serum and then assaying these samples (Tables III–V). The ratios of assayed to added indicated by their proximity to unity that the accuracy of the assay was at an acceptable level. The precision was also acceptable in that the standard errors of the replicates were 5% or less of the mean value for each concentration measured.

The validity of the assay was further established by comparing the values obtained by radioimmunoassay to values obtained from a ^{14}C-oxfendazole radiochemical assay. The results (Table VI) indicated a relatively good agreement. Most of the values obtained by the two assays were within 20% of one another. The difference in the results was probably due to variability in the TLC procedure.

Table VI—Comparison of Oxfendazole Levels as Measured by Radioimmunoassay and ^{14}C-Radiochemical Assay

Calf	Postdose Time, hr	Plasma Oxfendazole Levels, ng/ml	
		Radioimmunoassay	^{14}C-Radiochemical
1	2	226	203
	4	376	310
	8	447	337
	24	209	258
	48	102	171
2	2	195	185
	4	266	246
	8	269	286
	24	188	224
	48	80	150
3	2	250	271
	4	400	354
	8	443	365
	24	257	291
	48	74	143

Plasma Concentration in Experimental Animals—Plasma concentration–time profiles were determined in the calf after administering an oral dose of oxfendazole (Fig. 3). The profile indicated that the method has sufficient sensitivity to be useful in a variety of oral or intravenous experiments. Similarly, the profiles obtained in dogs showed that the sensitivity was adequate. The peak plasma levels achieved after 11- and 33-mg/kg doses varied between 1100 and 1400 ng/ml, respectively.

In summary, the method is sensitive, specific, and relatively simple. Analytical measurements can be performed directly on an aliquot of plasma without prior extraction or purification.

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