High-Pressure Liquid Chromatographic Analysis of Veterinary Anthelmintics I: Quantitative Determination of Tetramisole

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Abstract □ High-pressure liquid chromatography was used with a 10-μm C_8-type bonded silica gel column to quantitate the tetramisole in veterinary anthelmintic preparations. The chromatographic elution time was less than 10 min, and other compounds present in the products analyzed did not interefere.

Keyphrases □ Tetramisole—high-pressure liquid chromatographic analysis, veterinary preparations □ High-pressure liquid chromatography—analysis, tetramisole in veterinary preparations □ Anthelmintics—tetramisole, high-pressure liquid chromatographic analysis in veterinary preparations

Tetramisole (*dl*-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-*b*]thiazole) hydrochloride (1) is a potent, broad-spectrum anthelmintic; it exhibits high activity against several GI and pulmonary nematode species. Tetramisole is often used in veterinary therapeutics, either alone or with other anthelmintics such as bithionol, bithionol sulfoxide, and oxyclozanide.

UV spectrophotometry cannot be used directly for identifying tetramisole because the tetramisole absorption maximum (214 nm) is shared by other organic substances. TLC separation of tetramisole from its thermal degradation products was reported (2) but was not quantitative. A partition column chromatographic method (3) resolved tetramisole from other interfering substances prior to UV measurement but was time consuming and unsuitable for routine analysis.

High-pressure liquid chromatography (HPLC) offers a viable alternative for the quantitative analysis of tetramisole in commercial products and is applicable to various formulations.

EXPERIMENTAL

Equipment—The liquid chromatograph[1] was operated at ambient temperature and was equipped with a UV detector for monitoring the column effluent at 254 nm. The stainless steel column, 15 cm × 4.7 mm (i.d.), was packed with a C_8-type permanently bonded silica gel[2]. The liquid chromatographic detector was connected to a computing integrator[3] for obtaining the chromatogram peak areas.

Reagents—Tetramisole hydrochloride[4] and phenol[5] were the reference and internal standards, respectively.

Analytical reagent grade acetonitrile[5] and sulfuric acid[5] were used for the mobile phase preparation.

Mobile Phase—A mixture of 1% concentrated sulfuric acid in water–acetonitrile (80:20 v/v) was used.

Internal Standard Solution—Accurately weighed phenol (~500 mg) in a 100-ml volumetric flask was dissolved in, and diluted to volume with, the mobile phase.

Standard Solution—Accurately weighed tetramisole standard (~100 mg) in a 100-ml volumetric flask was dissolved in, and diluted to volume with, water. A tetramisole standard–phenol solution mixture (1:1) in a suitable vessel was mixed well.

Sample Solution—An anthelmintic sample equivalent to 100 mg of tetramisole was pipetted accurately into a 100-ml volumetric flask and diluted to volume with water. A 1:1 sample solution–phenol solution mixture in a suitable vessel was mixed well.

Formulations Containing Bithionol and Oxyclozanide—Veterinary formulations containing tetramisole–bithionol or tetramisole–oxyclozanide are suspensions. The formulation was mixed thoroughly, and an aliquot equivalent to 100 mg of tetramisole was pipetted quickly into a 50-ml glass-stoppered centrifuge tube. Water (25 ml) was added, and the solution was mixed thoroughly and centrifuged at 2000 rpm for 5 min. The supernate was transferred into a 100-ml volumetric flask, and the residue was extracted with 4 × 10 ml of water. The combined extracts in the 100-ml flask were diluted to volume with water.

Chromatography—The column was conditioned 0.5 hr with mobile phase (flow rate of 1.6 ml/min). Four-microliter standard solution aliquots were injected onto the column, and the tetramisole (2.4 min) and phenol (6.0 min) peaks were identified. The relative area response ratio was determined by replicate standard solution injections. When the response ratio deviation was less than 2%, the sample dilution was injected.

Linearity—The method was evaluated for tetramisole response linearity at concentrations ranging from 0.02 to 2% of tetramisole. Four microliters of each solution was injected into the chromatograph, and the tetramisole–internal standard area ratio was calculated and plotted *versus* the tetramisole concentration. The chromatographic response was linear up to the highest tetramisole concentration tested (2%).

RESULTS AND DISCUSSION

The results (Table I) show that tetramisole can be assayed in veterinary anthelmintic formulations using a simple HPLC method. However, these results could not be tested against an alternative (tested or official) method because no such method exists. The HPLC analyses gave a linear response with tetramisole concentrations between 0.02 and 2%. An aqueous 0.1% tetramisole standard was assayed with each batch of samples, and the sample concentration calculations were based upon this standard.

The commercial anthelmintic preparations generally contained bithionol, bithionol sulfoxide, or oxyclozanide in addition to tetramisole, but these agents did not interfere with the tetramisole assay since they were not eluted under the conditions described. Bithionol, bithionol sulfoxide, and oxyclozanide remaining on the column were subsequently eluted by changing the mobile phase to acetonitrile (1 ml/min) and eluting for 20 min after every series of 20 samples had been run.

Although 254 nm is the most commonly used wavelength, tetramisole elution was monitored at its absorption maximum (214 nm); detection

Table I—Percent Tetramisole Concentration in Anthelmintic Veterinary Formulations

Sample	Labeled Tetramisole	Found Tetramisole[a]
1[b]	7.5	7.9 ± 0.1
2[c]	3.4	3.4 ± 0.05
3[d]	3.4	3.51 ± 0.05
4	3.4	3.48 ± 0.05
5[e]	3.4	3.46 ± 0.05

[a] Mean of three sample preparations and standard error. [b] Containing tetramisole free base. [c] Containing oxyclozanide (3.4%). [d] Containing bithionol (8%). [e] Containing bithionol sulfoxide (10%).

[1] Varian 8500.
[2] Lichrosorb RP8, 10 μ, Merck & Co.
[3] System I, Spectra-Physics.
[4] Lot CA 76.007.00, Specia, Paris, France
[5] Merck & Co.

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at 214 nm was eightfold more sensitive and would be useful in residue analysis.

REFERENCES

(1) D. Thienpont, O. F. J. Vanparijs, A. H. M. Raeymaekers, J. Vandenberk, P. J. A. Demoen, F. T. N. Allewijn, R. P. H. Marsboom, C. J. E. Niemegaers, K. H. L. Schellekens and P. A. J. Janssen, *Nature*, **209**, 1084 (1966).

(2) N. A. Dickinson, H. E. Hudson, and P. J. Taylor, *Analyst*, **96**, 235 (1971).

(3) *Ibid.*, **96**, 244 (1971).

Possibility for Error in FDA Diffusion Assays

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Received September 26, 1978, from *Eli Lilly and Company, Indianapolis, IN 46206.* Accepted for publication November 20, 1978.

Abstract □ Computational procedures specified for the FDA single-dose diffusion assay for antibiotics may cause substantial error in estimated sample potency. An unrecognized mistake in reference solution concentration is the source of error. It is caused by correcting responses from standard and sample plates differently. The error can be avoided by correcting both standard and sample responses to the observed reference response.

Keyphrases □ FDA—assay, antibiotics, correction □ Antibiotics—analysis, FDA diffusion assay, corrections

The single-dose diffusion assay of the Food and Drug Administration (FDA) has been a satisfactory antibiotic assay for more than 30 years. The method is about as accurate as any other diffusion method.

Since samples and standards are on different petri plates, sample responses must be corrected for variations in zone sizes attributable to plates. To this end, one-half of the responses on sample plates are from a reference solution, which is also the middle concentration of the standard line.

The correction procedures specified in the FDA regulations (1) are different for standards and samples and can cause substantial error under certain circumstances. Causes of errors, their sizes, and procedures for avoiding them will be discussed.

DISCUSSION

The standard line used in this assay was relatively short to avoid complication caused by excessive curvature. Standard concentrations did not follow a 1.35 multiplier exactly but were adjusted to concentrations that could be prepared accurately using volumetric pipets and flasks. The standard lines were computed by linear regression analysis and not by the modified least-squares formula of the "Code of Federal Regulations" (CFR) 436.105 (1).

The data for this investigation were obtained from a *Bacillus subtilis* (ATCC 6633) pen-cylinder agar diffusion assay for cephalexin. For the assay, plastic petri plates (100 × 15 mm) contained a single 10-ml layer of Medium 1 (2) seeded with *B. subtilis* spores. Standard and sample solutions were made from a freshly prepared stock solution in pH 6.0 buffer. Standards containing 2.00, 2.75, 3.75, 5.00, or 6.50 μg of cephalexin activity/ml and samples containing 2.75, 3.00, 4.00, or 6.00 μg of activity/ml were applied at a dosage of 0.2 ml/cylinder. The solution used for the 2.75-μg/ml sample was the same solution used for the 2.75-μg/ml standard. Plates were incubated at 30° for 16–18 hr, and inhibition zones were measured to the nearest 0.01 mm with an antibiotic zone reader[1] as described elsewhere (3).

Zone diameters are given in Table I. A standard line graph is given in

Table I—Standard and Sample Responses

Standard, μg/ml	Response, mm	
	Standard	Reference
2.00	14.25	16.14
2.75	15.71	16.08
(3.75)	(16.12)	—
5.00	18.12	16.07
6.50	19.31	16.18
	Response, mm	
Sample	Sample	Reference
1	16.12	16.19
2	19.14	16.31
3	17.21	16.01
4[a]	15.20	15.61

[a] The plates for this sample were purposely incubated at a temperature slightly different from the standard plates.

Fig. 1. Estimated potencies computed as required by the CFR 436.105 (1) and by two modifications are given in Table II. All potencies were interpolated from the best straight line through the standard responses. The reference standard was purposely made at 3.0 μg/ml and not of the 3.75 μg/ml its label indicated. This fact placed a small bias in all potencies, as could be inferred from Fig. 1.

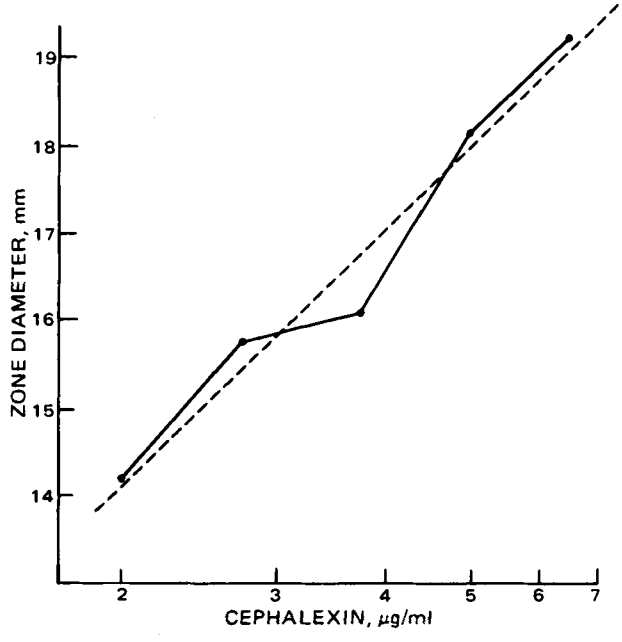

Figure 1—*Calibration line for an assay of cephalexin by* B. subtilis *(ATCC 6633). The true concentration of the reference solution (3.75 μg/ml) point is 3.00 μg/ml. The dashed line is the least-squares best line through the five points.*

[1] Fisher-Lilly.

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