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

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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 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.

[1] Fisher-Lilly.

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.

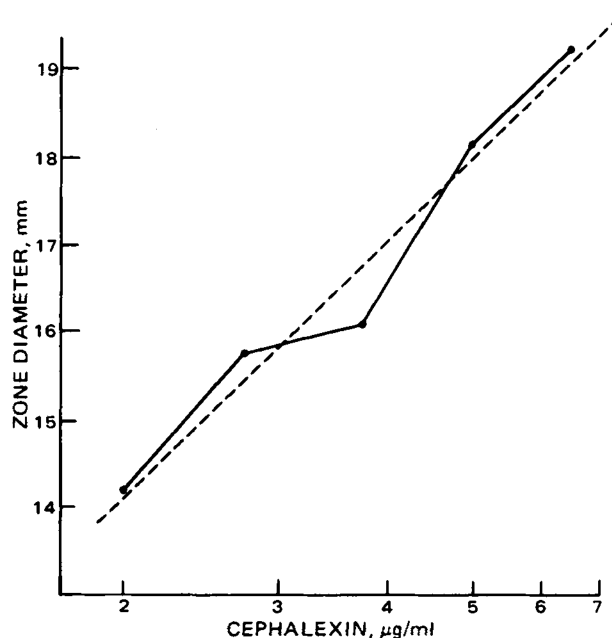

Figure 1—*Calibration line for an assay of cephalexin by* B. subtilis *(ATCC 6633). The true concentration of the reference solution (3.75 μg/ml) point is 3.00 μg/ml. The dashed line is the least-squares best line through the five points.*

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Table II—Potencies Computed by FDA and Lilly Procedures from the Best Straight Lines

Sample	True Concentration, μg/ml	Five-Point Line, Including Reference Point		Four-Point Line Excluding Reference Point, Lilly
		FDA	Lilly	
1	3.00	3.69	3.15	3.02
2	6.00	7.36	6.27	6.02
3	4.00	4.99	4.26	4.09
4	2.75	3.40	2.90	2.79
Reference, mm		16.79	16.12	16.12
Mean computational error, %		+24	+5	0

Standard responses were corrected to the observed mean reference concentration response ("3.75" μg/ml) on all standard plates. Sample plate responses were corrected to the theoretical reference response, the 3.75-μg/ml point on the best straight line through the five standards as required by regulations (1). These results are given in the "FDA" column of Table II.

A second set of potencies was obtained from the same data by correcting sample zones to the observed reference as was done for standards and interpolating from the same straight line. These results are given in the "Lilly" column of Table II. A third set of potencies was obtained by correcting to the observed reference and by rejecting the obviously erroneous reference response when computing the best straight line. These results were taken as the true sample assay.

The computational error of +24% in the FDA procedure was caused by the computed reference diameter required by the CFR (1). The error caused by including the erroneous reference point in the calculation of the best straight line was about 5%.

The plates for Sample 4 were incubated at a temperature slightly different from that of the standard plates. Since each response was corrected to the reference concentration, this difference in temperature and the resultant reduction in zone diameters had no effect on the calculated sample potency.

The correction procedure of Ref. 1 is without error only when the observed mean reference diameter is identical with that interpolated from the best straight line. Since this is an unlikely occurrence, a different correction scheme should be used. Correcting both standard and sample responses to the response observed for the reference and then calculating estimated potencies from the regression equation will avoid the errors of the CFR (1).

Proper plate correction is of more than academic interest because computational errors as great as 40% have been reported from other laboratories[2]. Such an error was caused by using a reference about three-fourths as concentrated as it was thought to be. Large errors seem to be rare, but smaller ones are not. In one series of 40 response lines from 16 laboratories, seven of the reference zones, including the one just mentioned, were obviously in error and nine other standard zones had errors. Except for the reference, errors were evenly distributed among the other four standards. Three calibration lines had two obvious errors in them. All of these lines were used in the laboratories reporting the work.

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[2] Dr. H. S. Ragheb, Department of Biochemistry, Purdue University Agricultural Experiment Section, West Lafayette, IN 47907, personal communication.

High-Pressure Liquid Chromatographic Feprazone Determination in Pharmaceutical Formulations

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Received September 12, 1978, from the *Istituto di Farmacologia, Università di Ferrara, 44100 Ferrara, Italy.* Accepted for publication November 30, 1978.

Abstract □ A high-pressure liquid chromatographic method was developed for the quantitative determination of feprazone, a nonsteroidal anti-inflammatory agent, in different pharmaceutical formulations. The results agree with those obtained with GLC and UV spectrophotometric assays.

Keyphrases □ High-pressure liquid chromatography—analysis, feprazone in various pharmaceutical formulations □ Feprazone—analysis, high-pressure liquid chromatography, various pharmaceutical formulations □ Analgesics—feprazone, analysis in various pharmaceutical formulations, high-pressure liquid chromatography

Feprazone (4-prenyl-1,2-diphenyl-3,5-pyrazolidinedione) is a nonsteroidal analgesic, antipyretic, and anti-inflammatory agent with a low ulcerogenic potential. Feprazone has been determined in pharmaceutical formulations and in body fluids by potentiometric titration (1), UV spectrophotometry (2), TLC (3), GLC (4–6), and radioisotopic techniques (7).

This paper describes the quantitative determination of feprazone in capsule, suppository, and cream formulations by a simple and rapid high-pressure liquid chromatographic (HPLC) procedure.

The proposed HPLC method was compared to GLC (5) and UV spectrophotometric (2) assays.

EXPERIMENTAL[1]

HPLC—*Equipment and Operating Conditions*—A liquid chromatograph[2], equipped with a UV-visible detector[3] and with a septum injector[2], was used. The column[4] was 50 cm × 2.2 mm i.d., and the microcell[5] volume was 8 μl. The flow rate was 0.49 ml/min under a constant pressure

[1] All solvents were BDH AnalaR grade and were used without further purification.
[2] Series 4000, Varian Aerograph, Palo Alto, Calif.
[3] Beckman DU, Beckman Instruments, Fullerton, Calif.
[4] Micropak SI 10, Varian Aerograph, Palo Alto, Calif.
[5] Circus, Milan, Italy.

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