

Semiautomated Turbidimetric Microbiological Assay for Determination of Cefazolin

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The Autoturb System, a semiautomated system for photometric bioassay, was used to determine cefazolin content. Suitable conditions for the assay using *Streptococcus faecalis* ATCC 10541 as the indicator organism included a medium pH of 6.0 to 7.0 and an incubation time of 3 to 3.5 h at 36 C. Multiple independent assays of samples from a common batch showed the test to be highly reproducible. Accuracy of the turbidimetric assay was evaluated by comparing data obtained from a chemical (hydroxylamine) and a biological (disk diffusion) assay. The available data show the turbidimetric assay to be a rapid, accurate, and reproducible method for determining the biological activity of cefazolin samples.

Cefazolin (Fig. 1), a new cephalosporin antibiotic currently under development in our laboratory, has a broad spectrum of antibacterial activity (3-6). Standard plate diffusion assays for antibiotics, although adequate, often do not provide the rapid and accurate assay information for the large number of test samples generated by development and control needs. Excellent precision can be achieved with a manual turbidimetric microbiological assay provided special care is taken in all of the operational details of the assay (1). A system for carrying out semiautomated photometric assay, the Autoturb System, has been reported to give a high order of precision for the assay of vitamins and antibiotics including cephalixin (2). This system has also been used for carbenicillin assay, resulting in increased speed and precision over the plates diffusion assay (7).

The present report details our experience with the use of the Autoturb System for the assay of cefazolin. Included in the study was the effect of culture conditions and a comparison with other assay procedures.

MATERIALS AND METHODS

Cefazolin. Samples were in the acid or sodium salt form. Solutions were prepared daily in 1% phosphate buffer, pH 6.0. The cefazolin assay standard was kept in a desiccator at -20 C until used.

Culture conditions and media. A *Streptococcus faecalis* (ATCC 10541) culture (obtained from T. Platt, Squibb Institute for Medical Research, New Brunswick, N.J.) was used for the assays. It was incubated overnight at 30 C in micro inoculum broth (Difco Laboratories), centrifuged, washed once and then

resuspended in pH 6 buffer, and frozen at -70 C until used for the inoculum. The inoculum was used at a concentration of approximately 4×10^6 colony-forming units per ml of medium. A frozen inoculum can be used successfully after storage for at least 15 months. A fresh culture is also satisfactory for use. Assay medium contained: glucose (2%); yeast extract (0.5%); KH_2PO_4 (0.1%); K_2HPO_4 (0.1%); sodium citrate (1%); and Trypticase (0.9%). The pH was 6.8 before autoclaving for 15 min at 121 C, and remained essentially unchanged after autoclaving. Medium was used at room temperature.

Turbidimetric assays. The Autoturb, a semiautomated system for doing photometric assays described by Kuzel and Kavanagh, was used in these studies (2). In use, a carousel holding 40 tubes provides media blanks, samples of known concentration, standards, and unknown samples for each of two self-contained independent assays. Tubes 1 to 20 comprise the first assay, and tubes 21 to 40 the second. Separate containers of inoculated media are used for each assay.

The Autoturb Sampler removes four separate measured samples from each carousel tube and dispenses each sample plus a measured volume of inoculated medium in separate assay tubes. In our assays, two sample volumes, in the ratio 2:3, were used. In effect, samples from each carousel tube were tested at two concentrations. A standard curve containing approximately 5, 7.5, 10, 15, 20, and 30 μg of cefazolin per ml of assay broth was included in each assay (carousel tubes contained 0.5, 1.0, or 2.0 mg of cefazolin per ml of buffer). Test samples were diluted in pH 6 buffer to a concentration of about 1.7 mg/ml before being placed in carousel tubes.

Inoculated tubes were incubated in a 36 C constant-temperature water bath until inoculum control tubes had a transmittance of 35 to 45% measured at 625 nm (about 3.25 h). After incubation, tubes were

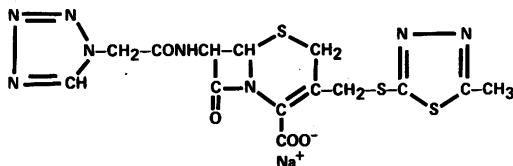


FIG. 1. Structure of cefazolin, 3-(5-methyl-1,3,4-thiadiazole-2-ylthiomethyl)-7-[2-(1H-tetrazol-1-yl)acetamidol]-3-cephem-4-carboxylic acid, sodium salt.

heated in a water bath at 80 C for 5 min to kill the culture and cooled to ambient temperature, and the transmittance was determined.

Transmittance was measured by the Autoturb Reader section and expressed in terms of millivolts (1,000 mV equals 100% transmittance) by using a modified Turner model 330 colorimeter equipped with a flow cell. The instrument was adjusted to read about 1,200 mV against buffer. The millivolt output was interfaced through an analogue to digital converter and required logic circuitry to a teletype paper tape punch. The resulting tape was loaded into an external timesharing computer for data analysis. Data was analyzed point to point by using a plot of transmittance versus concentration.

Plate assay. The disk agar diffusion assay employing petri dishes (15 by 100 mm) containing 7 ml of antibiotic medium no. 1 (Baltimore Biological Laboratory) inoculated with spores of *Bacillus subtilis*, ATCC no. 6633, was used. Six absorbent paper disks (Schleicher & Schuell Co. no. 740-E, 6.35 mm) containing either a test or reference solution were alternately placed on assay plates. Assays were incubated at 30 C for 18 h before determining the diameter of the inhibition zone using a Fisher-Lilly zone reader. After correction for reference variation, a dose-response curve was constructed using data from six assay plates at each of four cefazolin concentrations for both standard curve and unknown samples. The resulting regression lines were calculated by the method of least squares. Potency of the sample was determined by using a parallel-line bioassay. Two independent four-point assays were made for each sample. The 95% confidence limits on this assay are about $\pm 5\%$.

Chemical assay. The cefazolin content of the samples was determined chemically by means of an ultraviolet spectrophotometric procedure involving reaction with hydroxylamine. This procedure which has also been automated is to be published elsewhere. The method depends upon the well-known reaction between hydroxylamine and the β -lactam function of the cephalosporin nucleus to form a hydroxamic acid. This particular method differs from the conventional procedures in that no colored complex with ferric ion is required. This is due to the difference in the ultraviolet spectra of cefazolin and corresponding hydroxamic acid. The difference in absorbance between a reacted and an unreacted portion of a sample is a sensitive measure of intact cefazolin. At the 95% confidence limits, the variation in this assay is $\pm 2\%$ of the mean.

RESULTS

Effect of culture conditions. An example of the dose-response relationship using the described turbidimetric assay is shown in Fig. 2. In this figure, the growth response expressed in millivolts is plotted against cefazolin concentration. This plot shows a linear response between 4 and 18 $\mu\text{g/ml}$ over a 4.5-fold range. The incubation time was found to affect the slope of the dose-response (Fig. 3). Increased incubation results in a larger spread of growth response between the lowest and highest concentrations employed. On the basis of this response, a 3.5-h incubation time was selected for use in further studies. The effect of pH of assay medium on the growth response of *S. faecalis* to cefazolin is shown in Fig. 4. A growth response of 300 to 500 mV was observed in all media without cefazolin. The largest decrease in growth response was between pH 6 and 7. At pH 5 or 8, a relatively flat response was obtained, making these medium conditions unsuitable for assay. On the basis of data obtained, the most suitable pH range for the assay appears to be 6.0 to 7.0.

Comparison of the plate and turbidimetric assays. Potency of two seven-sample sets of sodium cefazolin was evaluated by using both plate and turbidimetric assays. Each sample was tested in two separate assays in each assay system. The averaged results for each set using each assay method are shown in Table 1. The 95% confidence limits of the two methods overlap to a large extent indicating no significant difference in the results obtained by the two

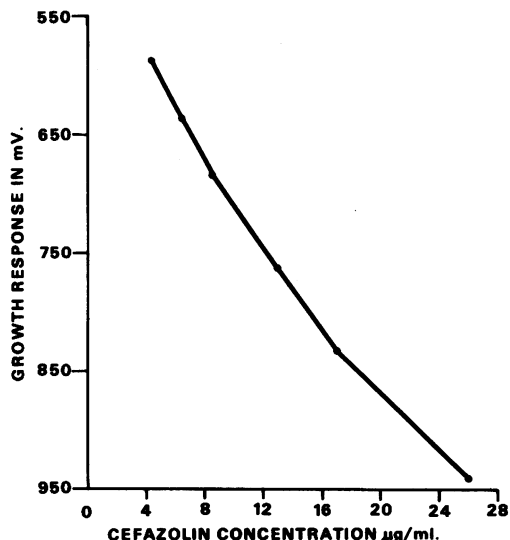


FIG. 2. Effect of cefazolin dose on growth of *S. faecalis* incubated in assay broth for 3 h.

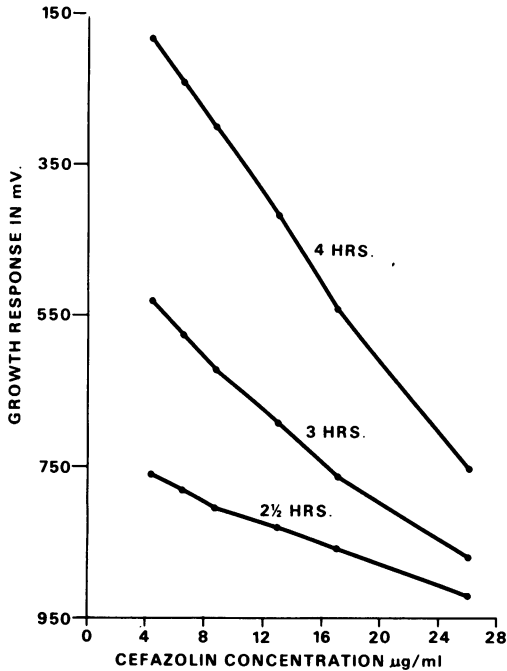


FIG. 3. Effect of incubation time on growth response of *S. faecalis* to cefazolin in assay medium, pH 6.8.

methods. The spread of the confidence limits is smaller using the turbidimetric assay, indicating a more reproducible test.

Assay reproducibility. Twenty-six weighings of a single sample of cefazolin-free acid were made and assayed using the turbidimetric assay over 4 days in a total of seven independent assays. These data show the turbidimetric assay for cefazolin to be highly reproducible. The spread between the lowest and highest potency estimates was less than 3% of the mean (Table 2).

Effect of sampling plan. Two lots, each containing three samples of sodium cefazolin, were examined by using a protocol designed to determine an efficient sampling plan. The study examined variation due to day-to-day effects, assay-to-assay effects, sample-to-sample effects, sample position, and sample size. None of these factors created significant bias in the results obtained. The data obtained showing day-to-day and sample-to-sample effects are summarized and statistically evaluated in Table 3. The effect of sampling plan on the confidence limits of the resulting potency estimates is given in Table 4. The result of this experiment suggested that two independent assays on each of one or two samples produce data as reliable as a more extensive sampling procedure. The largest increase in data reliabil-

ity was observed when more than one estimate was made. A single estimate produced data of reliability similar to that previously obtained using two independent plate diffusion assays.

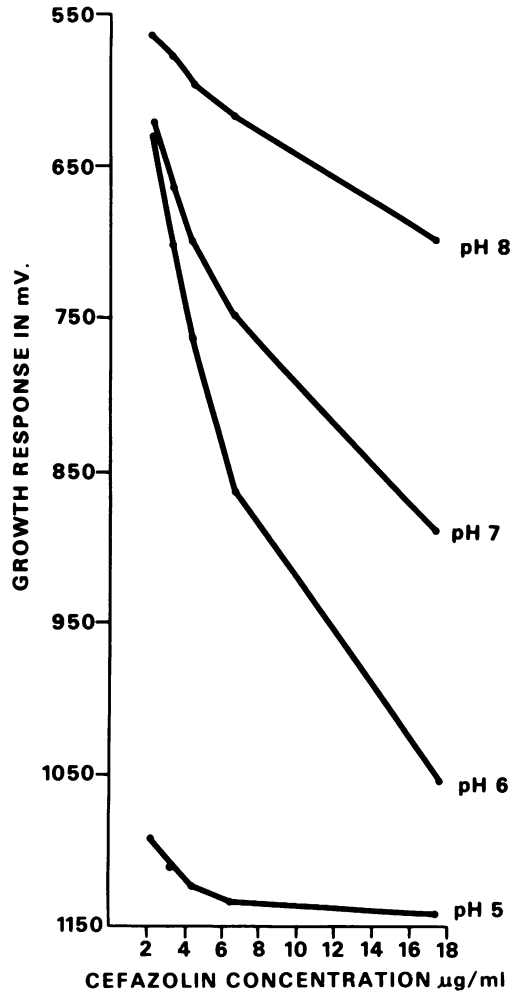


FIG. 4. Effect of media pH on growth response of *S. faecalis* to cefazolin. Tests were incubated until control tubes reached a transmittance of 35 to 45%.

TABLE 1. Comparison of plate disk agar diffusion and turbidimetric assays of two lots of sodium cefazolin

Lot	No. of samples	Assay ^a			
		Plate		Turbidimetric	
		Avg potency (µg/ml)	95% CL	Avg potency (µg/ml)	95% CL
A	7	955	926-984	927	914-940
B	7	922	894-950	917	899-933

^a CL, Confidence level.

TABLE 2. Reproducibility of turbidimetric assay by using a single lot of cefazolin-free acid^a

Potency tested on day						
1		2		3		4
A ^b	B	C	D	E	F	G
996	1,021	992	977	1,023	999	1,010
988	1,003	985	972	1,011	1,016	1,002
991	1,012	997	984			
988	996	1,008	995			
996	1,006					
985	989					

^a Potency is expressed in micrograms per milliliter. Average potency was 998 μ g; the 95% confidence limit was 972 to 1,024 μ g.

^b A through G are independent assays.

TABLE 3. Effect of sampling plan on reliability of cefazolin potency estimates by using the turbidimetric assay^a

Lot	Sample	Assay day	Potency	
			Mean	95% CL
1	A	1, 2, 3	945	936-953
	B	1, 2, 3	938	921-954
	C	1, 2, 3	939	930-948
	A, B, C	1	940	924-955
	A, B, C	2	951	943-959
	A, B, C	3	930	925-936
	A, B, C	1, 2, 3	940	934-947
2	D	1, 2, 3	930	913-946
	E	1, 2, 3	944	931-956
	F	1, 2, 3	931	920-942
	D, E, F	1	937	923-951
	D, E, F	2	948	934-962
	D, E, F	3	919	913-926
	D, E, F	1, 2, 3	935	927-943

^a Potency is expressed in micrograms of cefazolin per milliliter of free acid; CL, confidence limit.

Comparison of turbidimetric and hydroxylamine assays. Potency estimations obtained by using these two tests were compared to further evaluate the accuracy of the turbidimetric assay. The results were found to be essentially the same when using both tests. On the average, the turbidimetric assay showed the presence of 0.6% more cephalosporin content than did the hydroxylamine assay. The largest single difference between the two tests was 1.6% (Table 5).

DISCUSSION

A quantitative turbidimetric biological assay for cefazolin has been developed by using *S.*

faecalis ATCC 10541 as the indicator organism. Results obtained by using this assay are both reproducible and accurate and compare favorably with those obtained by using a plate diffusion or a hydroxylamine assay. At the 95% level of confidence, potency estimates obtained with the turbidimetric assays are ± 2 to 3%; plate diffusion assays ± 5 -6%; and the hydroxylamine assay ± 2 %. In addition to the advantage of greater accuracy, the automated turbidimetric assay offers several other advantages when compared with the manual plate diffusion assay. The turbidimetric assay is more rapid with results being obtained within the work day, whereas the plate diffusion procedure encompassed 2 working days. The number of samples processed by each operator increases at least fourfold with the Autoturb System.

The Autoturb assay for cefazolin compares favorably in terms of speed and accuracy with other antibiotic assays in which the Autoturb System is employed. Stankewich and Upton have recently reported 2 to 3% variation at the 95% level of confidence in potency estimations of carbenicillin by using *Escherichia coli* indicator and the Autoturb System (7). A similar low level of variation for Autoturb assay of penicillin G, erythromycin, and other antibiotics has been reported by Kuzel and Kavanagh (2). A

TABLE 4. Reliability of turbidimetric potency estimates for cefazolin by using various sampling protocols

No. of samples	No. of assays/sample(s)	95% CL ^a (Avg \pm %)
1	1	5
1	2	3
2	1	3
2	2	2
3	1	3
3	2	2

^a CL, Confidence limit.

TABLE 5. Comparison of the hydroxylamine and microbiological turbidimetric assay estimates of cefazolin content

Lot	Cefazolin content	
	Hydroxylamine	Bioassay
A	943	927
B	995	994
C	935	933
D	946	947
E	944	954
F	940	952
Avg	950.5	951.1

limitation of the Autoturb System is that it does not lend itself to assay of antibiotics in body fluids since a 10-ml sample size is required. We have had limited experience with Autoturb assay of cefazolin in urine samples where sufficient quantity is often available and found the assay to be feasible. In general, the system is most applicable to assay of bulk chemical and formulated samples.

The selection of an assay organism is important. A wide range of total growth in the control tubes will give satisfactory assays. The range depends upon characteristics of the test bacterium, composition of media, temperature, and time of incubation (2). The organism used in our system, *S. faecalis* (ATCC 10541), had the necessary combination of factors for this assay system. In addition, the inoculum remained stable for assay use upon freezing and could be used successfully after a period of at least 15 months of storage. Although this organism is not highly sensitive to cefazolin, it provides a wide working range (4.5-fold) needed for the interloop evaluation. Several assays were carried out with *Staphylococcus aureus*, a more sensitive indicator organism. The *Staphylococcus* assay responded to 2.5-fold lower concentrations than did the *Streptococcus* assay; however, the linear working range of the *Staphylococcus* assay was only 1.6-fold. This narrow working range made assays employing an interloop standard curve difficult to run reproducibly.

Evaluation of one or two samples in each of two independent turbidimetric assays appears to be an acceptable sampling plan for cefazolin. This plan yields potency estimates which are

± 2 to 3% at the 95% level of confidence. The turbidimetric assay for cefazolin described in this report has proven to be a useful and reliable measure of cefazolin potency in our laboratory. We have used this assay to determine the potency of more than 1,000 cefazolin samples and would recommend its use for any laboratory concerned with the need to assay large numbers of samples of bulk chemical or formulated materials.

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