SEMIAUTOMATED TURBIDIMETRIC MICROBIOLOGICAL ASSAY FOR CEFAZAFLUR

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A quantitative semi-automated turbidimetric bioassay for cefazaflur, using *Streptococcus faecium* as the indicator, is described. Assays were run at pH 6.5~7 for 3.75 hours at 37°C using 2~12 μg cefazaflur per ml assay broth for standards. The dose response line was plotted point to point using the natural log of the absorbance *vs* natural log of the concentration. This assay is both accurate and precise and is more rapid than traditional plate assays for antibiotics.

Cefazaflur, SK&F 59962, is a cephalosporin with a broad spectrum of *in vitro* and *in vivo* antibacterial activity. The pharmacokinetics have been studied in several species of laboratory animals and in man[1,2]. Plate assays for antibiotics, while acceptable, may not provide adequate sample throughout during development of a new compound. The Autoturb®, a semiautomated system for doing photometric assays, has been shown capable of producing large numbers of precise assays of vitamins or antibiotics including several β-lactams[3,4]. This system is best suited for the assay of bulk or formulated samples rather than samples of body fluids from clinical specimens. This paper details our experience with the Autoturb® for assay of cefazaflur. Included in the study are (1) dose-response relationship, (2) effect of pH, (3) effect of incubation time, (4) assay precision as a function of pH, (5) standard response line plotting parameters and (6) a comparison with two chemical assays.

Materials and Methods

Sodium cefazaflur samples were prepared fresh daily in 1% phosphate buffer, pH 6.0. The standard substance was kept in a dessicator at −20°C until used. The indicator culture used was *Streptococcus faecium* ATCC 10541. Culture conditions, media, and operation of the Autoturb® were described previously.[4] Separate standard response lines were prepared from data obtained from each of the four sampling loops of the Autoturb®. Results from test samples from each loop were calculated using the standard response line prepared from that loop. Data were analyzed point to point using a plot of the natural log of the absorbance *versus* the natural log of the concentration.

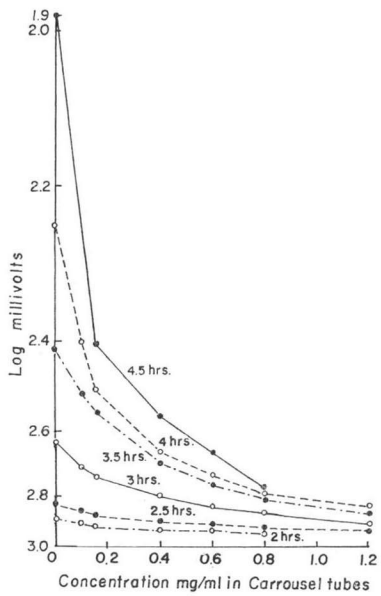
Chemical Assays: The cefazaflur content of samples was determined chemically using the automated hydroxylamine assay described for cefazolin[5] and by high pressure liquid chromotography (HPLC) described as follows: The HPLC assay was run at ambient temperature on a Chromatronix 3100 chromatograph using a strong anion-exchange resin column (DuPont 820960005) one meter long and 2.1 mm ID. The column was operated at 1,500 PSI and a flow rate of 1.3 ml/min. An ultraviolet detector operating at 254 nm was used. The mobile phase was 1.42% anhydrous sodium sulfate in distilled water adjusted to pH 3.5 with acetic acid. Samples were introduced to the column using a 20 μl loop. Under these conditions, the retention time for cefazaflur was about 11 minutes.

Results and Discussion

Effect of Culture Conditions: An example of the dose-response relationship as a function of duration of incubation time is shown in Fig. 1. The growth response expressed in millivolts (1,000 mv equals 100% transmittance) was converted to natural logs and plotted against cefazaflur concentration. The duration of the incubation was found to affect the slope of the dose response line. Increased length of incubation results in a larger spread of growth response between the lowest and highest concentrations of cefazaflur. On the basis of this experience, a 3.75 ± 0.25 hour incubation period was selected for use in further studies. A useful dose response range of 0.2~0.8 mg per ml in the carrousel tubes (resulting in a final concentration of 2~12 μg/ml in the assay tubes) was observed. The effect of assay medium pH on the growth response of *S. faecium* is shown in Fig. 2. A growth response of 270~350 mv was observed at pH 6~8 in the absence of cefazaflur. The largest change in growth response, in the concentration range 0.2~0.8 mg/ml, was 250~290 mv and occurred at pH 6~7. On the basis of these data, the most suitable pH range for this assay appears to be 6~7.

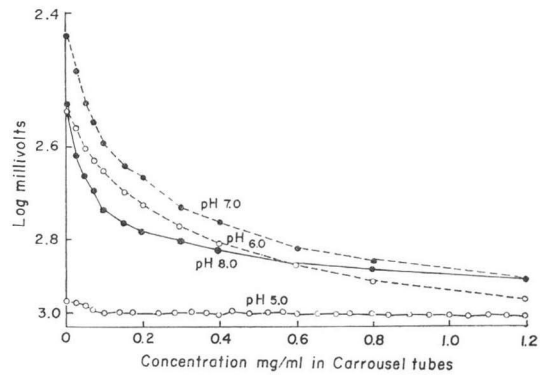
Assay Precision: Twelve weighings of a single lot of sodium cefazaflur were made and assayed 2 per day for a total of 6 days during a 2 week interval. The results of this study are shown in Table 1. All single estimates were within 3.9% of the overall average potency (945 μg/mg) and the average estimate from assays run on any one day was within 1.5% of this average. The precision of each potency estimate was further evaluated as a function of assay pH. Data in Table 2 show the best precision, as measured by the relative standard deviation, occurred when assays were run at pH 6.5~7.0.

Fig. 1. Effect of length of incubation cycle on growth response of *Streptococcus faecium* 10541 at pH 6.8 in the presence of graded concentrations of cefazaflur.



The effect of the method of plotting the standard response line on the apparent potency of known samples was evaluated. Five methods of data conversion were used: percent transmittance *vs* concentration; absorbance *vs* concentration; natural log absorbance *vs* concentration;

Fig. 2. Effect of medium pH on the response of *Streptococcus faecium* 10541 to graded concentrations of cefazaflur during 3.75-hour incubation.



natural log absorbance *vs* concentration squared; and natural log absorbance *vs* natural log concentration. Three data sets, each containing twelve potency estimates of solutions known to contain 400 μg active substance per ml were used for this analysis. A plot of natural log absorbance *vs* natural log concentration was uniformly more precise and accurate than the other plots. With this plotting method, the precision increased an average of 9.5~26.2% compared to the other data plots. The effect on the accuracy of the assay was less dramatic but noticeable (Table 3).

Table 1. Precision of the bioassay for cefazaflur potency expressed as mcg activity per mg weight

Assay day	Potency on test		Average
	1	2	
1	981	927	954
2	956	904	930
3	951	933	942
4	955	906	932
5	970	932	953
6	967	951	959
Range	951~981	904~951	930~959
Average	963	926	945

Table 2. Precision of the assay for cefazaflur as a function of assay pH as measured by the relative standard deviation of the individual potency estimates

Sample	Relative standard deviation at assay pH*					
	5.0	5.5	6.0	6.5	7.0	7.5
1	2.57	1.46	0.92	0.40	0.37	0.84
2	6.30	0.90	0.63	1.07	0.33	0.53
3	4.15	0.80	0.15	0.62	0.41	0.73
4	7.12	0.77	0.51	0.32	0.38	0.77
5	6.27	2.27	3.35	0.67	1.68	0.90
6	5.91	1.89	2.53	0.73	1.73	1.13
7	5.85	1.22	0.53	0.52	0.43	0.77
8	6.30	0.82	0.24	0.17	0.55	0.38
Average	5.55	1.25	1.07	0.56	0.73	0.75

* Relative standard deviation (RSD)$=(100/P_a\sqrt{E-1})\sqrt{(P_a-E_1)^2+(P_a-E_2)^2+(P_a-E_3)^2+(P_a-E_4)^2}$

P_a = Average potency of sample

E = Number of potency estimates

$E_{1\sim4}$ = Individual potency estimates

Table 3. The effect of standard response line plot type on the precision and accuracy of the assay

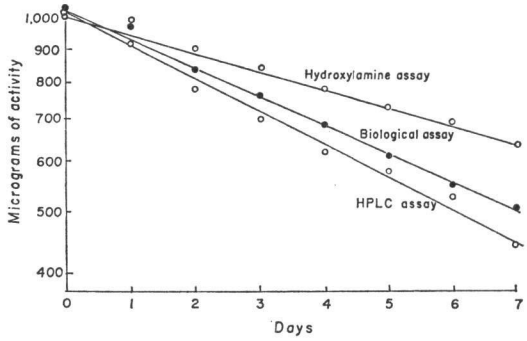
Parameter		Plot type				
		T: C	A: C	Log A: C	Log A: C^2	Log A: Log C
Precision	Range RSD[a]	0.14~1.42	0.12~1.46	0.14~1.26	0.12~1.44	0.16~1.35
	Average RSD	0.663	0.660	0.577	0.580	0.525
Accuracy	Range[b]	394~406	394~406	393~405	395~406	390~404
	Average	400.9	401.1	400.5	401.2	399.6

[a] See footnote Table 2. [b] μg activity recovered.

Comparison of Turbidimetric and Chemical Assays: The contents of a vial containing 1 gram of sodium cefazaflur were dissolved in 1 liter of pH 6 buffer and held at 37°C. Samples were withdrawn immediately after preparation and daily for 1 week. All samples were evaluated for

their content of sodium cefazaflur using the turbidimetric, automated hydroxylamine and HPLC assays. The results of this study are shown in Fig. 3. It is immediately obvious that the three assays give equivalent initial potency estimates but they diverge as the potency of the samples decline. The hydroxylamine assay appears to give a uniformly higher response, whereas the HPLC assay gives a lower response than the turbidimetric assay. The points used to produce the regression line for the hydroxylamine and for the turbidimetric assays have little scatter whereas those from the HPLC assay have larger scatter. The tendency of the hydroxylamine assay to produce higher potency estimates than the turbidimetric and HPLC assays was subsequently verified in additional experiments. It would appear that storage at 37°C results in biologically inactive degradation products that are measured by the hydroxylamine but not by the biological and HPLC assays.

Fig. 3. Comparison of biological, hydroxylamine and HPLC assays in evaluating the stability of cefazaflur dissolved in pH 6 buffer and held at 37°C.



A quantitative turbidimetric bioassay for cefazaflur has been developed using growth of *S. faecium* as the indicator. Results obtained using this assay are both precise and accurate. Potency estimates obtained with this turbidimetric assay are within $\pm 2 \sim 3\%$ at the 95% confidence level whereas conventional plate assays give values with deviations of $\pm 5 \sim 6\%$ or more. In addition to the increase in precision compared to plate analysis, the turbidimetric assay is more rapid and allows results to be obtained the same day as tests are run whereas plate assays require an extra day. This assay could be used readily by any laboratory concerned with the need to assay large numbers of samples of bulk or formulated cefazaflur.

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