# Iodometric and Spectrophotometric Assays for Cephradine after Its Hydrolysis with a β-Lactamase

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**Abstract** □ Iodometric and spectrophotometric methods were developed for the analysis of the new antibiotic cephradine. The procedures are modifications of known methods but are novel in employing a specific β-lactamase for hydrolysis of the β-lactam ring of the cephalosporin molecule. The iodometric method is rapid, precise, and accurate, but it requires fairly large amounts of cephradine. The spectrophotometric method, using differential UV absorption at 260 nm, is more rapid and more sensitive than the iodometric method but somewhat less accurate. Both methods proved useful for the routine assay of cephradine in certain formulations.

**Keyphrases** □ Cephradine—iodometric and spectrophotometric analyses, pharmaceutical formulations □ Iodometry—analysis, cephradine, pharmaceutical formulations □ Spectrophotometry—analysis, cephradine, pharmaceutical formulations □ Antibiotics—cephradine, iodometric and spectrophotometric analyses, pharmaceutical formulations

Many assay methods for the cephalosporins have been developed from procedures originally utilized for the assay of penicillins and have been based primarily on the presence of the β-lactam ring in both classes of antibiotics. Although microbiological assays using agar diffusion (1) or photometric (2) procedures are preferred for the determination of cephalosporins in pharmaceutical preparations, body fluids, and formulations being studied for long-term stability, they are imprecise and tedious.

Chemical methods commonly used for the assay of β-lactam antibiotics are based on determining the products formed after hydrolysis of the β-lactam ring. The methods include an iodometric procedure (3), the hydroxylamine procedure (4), a manometric assay (5), and an alkalimetric method (6). All of these chemical methods can be performed rapidly but sometimes lack the specificity of the microbiological assays. This lack of specificity is particularly true of the hydroxylamine method, since many amides, anhydrides, esters, aldehydes, and ketones react with hydroxylamine and give rise to erroneous results.

The iodometric method, which is based on the measurement of the penicilloic acid formed upon alkaline hydrolysis of penicillin, is more specific. Direct spectrophotometric methods for cephalosporins (7) and, more recently, for penicillin (8) have been reported but have not been used extensively in the routine analysis of these antibiotics.

Although Alicino (9) successfully applied the iodometric assay to the determination of cephalosporin C, attempts in this laboratory to apply this method to the determination of cephradine {7-[D-(−)-2-amino-2-(1,4-cyclohexadien-1-yl)acetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid} in formulations being studied for long-term stability gave erratic results. The recent development[1] in this laboratory of a β-lactamase (EC 3.5.2.6) with a high specificity toward cephradine and such related cephalosporins as cephalexin (7-[D-2-amino-2-phenylacetamido] - 3 - methyl-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid) prompted the development of an analytical method based on the iodometric procedure but using the enzyme for the specific hydrolysis of the β-lactam ring.

At the same time, a spectrophotometric assay for cephradine was developed, using the β-lactamase for hydrolysis. It is based on the assay described (7) for screening compounds for β-lactamase sensitivity and is similar to a published assay (10) in which the decrease in the characteristic UV absorption of the cephalosporin was determined after enzymatic hydrolysis.

[1] Unpublished data.

## EXPERIMENTAL

**Chemicals**—All chemicals were reagent grade. 7-Aminodeacetoxycephalosporanic acid[2], cephradine[3] used for standard solutions, and penicillin G potassium[4] were house products.

**Enzymes**—The β-lactamase, called cephradinase, was prepared essentially as described by Hennessey and Richmond (11), with the following exceptions: sodium β-glycerophosphate was omitted from the culture medium; the organism used was a species of *Enterobacter* originally isolated as a secondary infection from a cancer patient and now part of the Squibb Microbiology Culture Collection (SC 8415); the medium was inoculated with 1.0 ml of a 5.0-ml suspension of the organism washed from a slant of yeast beef agar with 0.1% peptone water; the inducer used was cephradine; and growth of the culture was continued for 67 hr.

The enzyme used in the assays was a crude preparation of the β-lactamase obtained by sonication of the cells in 0.1 *M* phosphate buffer, pH 8.0, and removal of the cell debris by centrifugation. The enzyme was diluted in 0.1 *M* phosphate buffer, pH 8.0. The unit of activity of this enzyme has been defined as the number of micromoles of cephradine hydrolyzed per hour by 1 ml of enzyme solution at room temperature (23–25°). Penicillinase concentrate[5], with an activity of $1 \times 10^6$ kinetic units/ml, was diluted 100 times in 0.1 *M* phosphate buffer, pH 7.0, before use.

**Preparation of Solutions**—Phosphate buffers and phthalate buffer were prepared as described previously (12). A 0.01 *N* sodium thiosulfate solution was prepared from a 0.1 *N* sodium thiosulfate solution that had been standardized against potassium iodate. A 0.0094 *N* iodine solution was prepared from a 0.1 *N* iodine solution and standardized by direct titration against the 0.01 *N* sodium thiosulfate solution.

**Methods**—All incubations were carried out at room temperature (23–25°). Unless otherwise stated, cephradinase (β-lactamase) solutions with activities of approximately 50 units/ml were used.

*Iodometric Assay Using Enzymatic Hydrolysis*—Cephradine samples or standards were prepared in 0.1 *M* phosphate buffer, pH 7.0, to give final concentrations of approximately 500 μg/ml (450-μg/ml potency). Duplicate 5.0-ml aliquots of a cephradine sample solution were incubated with 2.0 ml of cephradinase solution for 30 min. A third 5.0-ml aliquot was used as a control and was incubated with 2.0 ml of 0.1 *M* phosphate buffer, pH 8.0, for the same time.

[2] Lot 2005/C-1005, Squibb Chemical Development Laboratories, New Brunswick, N.J.

[3] Cephradine dihydrate, Lot NN005NB, biological potency 900 μg/mg, Squibb, New Brunswick, N.J.

[4] Potassium penicillin G-4 (1590 units/mg), Squibb, New Brunswick, N.J.

[5] Baltimore Biological Laboratories, Cockeysville, Md.

This procedure was repeated for a standard solution containing an accurately weighed, known amount of cephradine at a concentration of approximately 500 μg/ml (450-μg/ml potency). Then 10 ml of 0.3 *M* phthalate buffer, pH 4.5, and 10.00 ml of 0.0094 *N* iodine solution were pipetted into each flask. After each flask had been swirled to mix the contents completely, it was stoppered and left to stand in the dark for 30 ± 1 min. The solutions were then titrated with 0.01 *N* sodium thiosulfate, using 2 ml of starch solution as the end-point indicator.

The amount of cephradine in an unknown sample was calculated as follows:

$$\text{cephradine (mg)} = \frac{[\text{amount thiosulfate(control)} - \text{amount thiosulfate(sample)} \times \text{sample volume}]}{\text{iodine titer} \times 5 \times \text{sample weight (mg)}} \quad \text{(Eq. 1)}$$

where 5 = volume of sample or control in each flask, sample volume = volume of original sample solution, thiosulfate = 0.01 *N* sodium thiosulfate solution, and iodine titer = volume of 0.0094 *N* iodine solution consumed by 1 mg of cephradine after treatment with cephradinase. The iodine titer is determined from the titration values obtained with the *standard* cephradine solution and is calculated as follows:

$$\text{iodine titer (ml/mg)} = \frac{[\text{amount thiosulfate(control)} - \text{amount thiosulfate(sample)}]}{\frac{\text{standard weight (mg)} \times 5}{50}} \quad \text{(Eq. 2)}$$

Once the amount of cephradine in an unknown sample was calculated, the potency of the sample was obtained by multiplying the cephradine concentration obtained by the potency of the cephradine standard.

*Spectrophotometric Assay*—Cephradine samples or standard, prepared in water, were assayed in digests containing 1.5 ml of 0.01 *M* phosphate buffer, pH 8.0, which contained 0.001 *M* dithiothreitol, 0.5 ml of cephradinase solution (~10 units of activity), and cephradine solution (containing 10–100 μg, equivalent to a potency of 9–90 μg) in a total volume of 3.0 ml. Controls were set up for each sample or standard solution and contained 0.5 ml of cephradinase, which had been boiled for 30 min, in place of active enzyme.

The duration of incubation was determined by the activity of the enzyme preparation used and was always calculated to be greater than the time required for the enzyme to hydrolyze completely the maximum amount of cephradine estimated to be present in the samples to be assayed. As a rule, the activities of the enzyme preparations used in the assay were such that incubation times no longer than 45 min were required for the complete hydrolysis of the cephradine. The sample or standard cephradine solutions were always added last to the assay or control digests. The digests were then immediately mixed, covered, and left to incubate.

The absorbances of the sample and control digests were measured at 260 nm[6]. The instrument was initially adjusted to zero absorption with a blank assay digest, containing water instead of cephradine solution, in the reference cell and a blank control digest in the sample cell. Matched 1-cm silica cells with a spectral range of 220–2500 nm were used for the measurements. The absorbances of the standard and sample digests were then read in the same manner, with the assay mixture in the reference cell and the assay control mixture in the sample cell.

A standard curve of absorption *versus* amount of cephradine was prepared each day that the assay was performed, using standards ranging in concentration from 10 to 100 μg of cephradine/ml of water (9–90-μg/ml potency) prepared in water from pure cephradine dihydrate with a known potency of 900 μg/mg. Standards and sample solutions were always assayed in duplicate on the day of their preparation. The concentration of cephradine in an unknown sample was determined directly from the standard curve, and the potency of the sample was obtained by multiplying the concentration by the potency of the cephradine standard.

*Iodometric Assay Using Alkaline Hydrolysis*—This method, a direct modification of the method originally described by Alicino (9), was developed in these laboratories[7]. Cephradine samples and controls were prepared as described in the iodometric method using enzymatic hydrolysis; but instead of enzyme, the samples were incubated with 2 ml of 3 *N* sodium hydroxide solution for 30 min. Then 2 ml of 3 *N* hydrochloric acid was added to the sample mixtures. No additions were made to the controls.

Then 10 ml of 0.3 *M* phthalate buffer, pH 4.5, and 10.00 ml of 0.0094 *N* iodine solution were added to each flask; the flasks were left to stand in the dark for 30 ± 1 min and were then titrated with 0.01 *N* sodium thiosulfate solution, as described previously. Cephradine potencies were calculated as they had been after enzymatic hydrolysis.

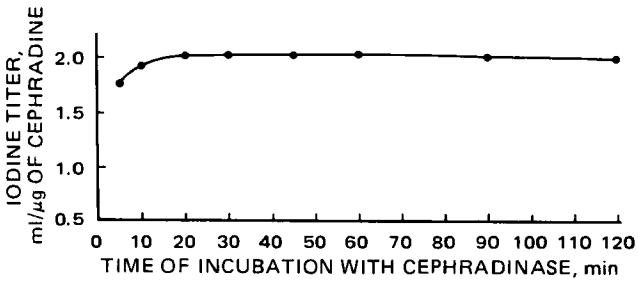


**Figure 1**—*Iodine titer* versus *time of incubation with cephradinase. Standard solutions of cephradine, with concentrations of approximately 500 μg/ml, were incubated with 2.0-ml aliquots of cephradinase for the periods shown and were then treated with iodine for 30 ± 1 min and titrated with sodium thiosulfate solution as described under* Methods.

## RESULTS

**Iodometric Assay**—Certain experiments were carried out to determine the optimum conditions for running the assay.

Since the iodine titer, required for the calculation of cephradine concentration in unknown samples, was determined directly from the cephradine standard assayed at the same time as the unknown samples, the construction of a standard curve was unnecessary. The iodine titer was found to have almost the same value from day to day and varied only slightly as a result of slight changes in the concentration of the iodine and sodium thiosulfate solutions used in the iodometric procedure.

The iodine titer was affected, however, by the duration of incubation of cephradine with the cephradinase. This effect was investigated in an experiment in which a standard solution of cephradine, with a concentration of 569 μg/ml in 0.1 *M* phosphate buffer, pH 7.0, was incubated for various times with the enzyme at room temperature. Controls were incubated with 0.1 *M* phosphate buffer, pH 8.0, in parallel with the samples. At the end of each incubation period, samples were assayed as already described.

Figure 1 shows that for the particular enzyme preparation used in this experiment (activity of 47.7 units/ml), the iodine titer increased with the duration of incubation with cephradinase up to 20 min, remained constant until 60 min, and then declined slightly until 120 min. Therefore, for routine determinations of cephradine in solutions containing the drug in concentrations of approximately 500 μg/ml, a cephradinase preparation with an activity of approximately 50 units/ml was sufficiently active to bring about complete hydrolysis of the β-lactam substrate within 30 min without any change in iodine titer.

*Optimal Time of Iodination*—Although the original iodometric method for the assay of penicillin preparations described by Alicino (3) required the incubation of the hydrolyzed β-lactam compound with excess iodine for 30 min, the more recently described procedure (13) requires only 15 min of incubation with excess iodine. This difference led to attempts to determine the optimal time of iodination for cephradine hydrolyzed by cephradinase.

Standard solutions of cephradine in 0.1 *M* phosphate buffer, pH 7.0, with a concentration of approximately 500 μg/ml, were incubated with the enzyme for 30 min. Controls, incubated with 0.1 *M* phosphate buffer, pH 8.0, were run in parallel. After the 30-min incubation period, all digests were treated with 10 ml of 0.3 *M* phthalate buffer, pH 4.5, and 10.00 ml of 0.0094 *N* iodine solution. After rapid and thorough mixing, the iodinated solutions were left in the dark at room tem-

[6] Beckman DB recording spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

[7] F. M. Russo-Alesi, personal communication.

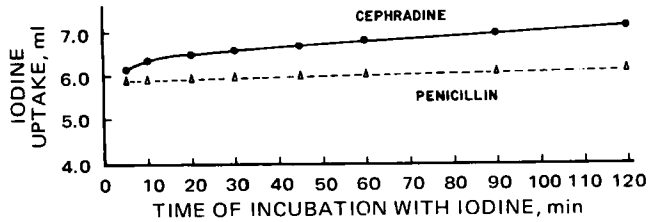


**Figure 2**—*Effect of time of iodination on iodine uptake. Standard solutions of cephradine, with concentrations of approximately 500 μg/ml, were incubated with cephradinase for 30 min. They were then treated with 10 ml of phthalate buffer and incubated with 10.00 ml of 0.0094* N *iodine solution for the periods shown. They then were titrated with sodium thiosulfate solution as described under* Methods. *Exactly the same procedure was followed for penicillin, except that the hydrolyzing enzyme was penicillinase.*

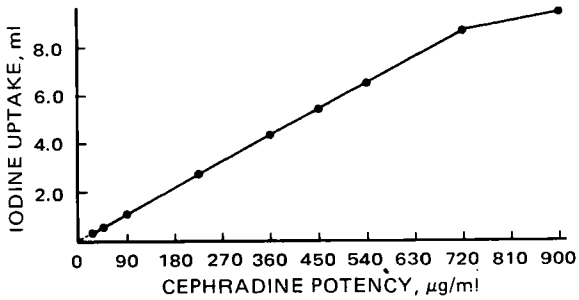


**Figure 3**—*Limits of the iodometric assay for cephradine using cephradinase for hydrolysis. Standard solutions of cephradine, with the potencies shown, were hydrolyzed with cephradinase and assayed by the iodometric procedure as described under* Methods.

perature for various intervals, which were precisely timed with a stopwatch. Thereafter, the iodine uptake was determined by titration with sodium thiosulfate, as described.

Figure 2 shows that when cephradinase was used to hydrolyze the β-lactam ring, the increase in iodine uptake with time was very slow after the first 20 min of iodination. Iodine uptake continued to increase as the iodination time progressed and did not reach a constant value. The same figure shows iodine uptake *versus* time of iodination for penicillin G potassium hydrolyzed by penicillinase. Here, too, iodine uptake increased slowly as the iodination time progressed, but the increase was slower and iodine uptake appeared to remain constant after 90 min of iodination. Since iodine uptake for cephradine hydrolyzed by cephradinase increased slowly with an increase in iodination time, the standard period of iodination, established as 30 ± 1 min, was timed precisely with a stopwatch.

*Precision*—The precision of the procedure was determined from analyses of solutions of cephradine made on 3 consecutive days by two operators. Standard solutions of cephradine in 0.1 *M* phosphate buffer, pH 7.0, approximately 500 μg/ml (450-μg/ml potency), were prepared on each day of assay and were used by both operators. A single sample solution of cephradine prepared in the same buffer, approximately 500 μg/ml (450-μg/ml potency), was prepared on each day of assay and was used by both operators.

Each operator assayed the sample solution 10 times each day, with controls being run in parallel for each two sample assay digests. The results (Table I) showed good reproducibility. For the 60 determinations, the recovery was 99.3%, the standard deviation of the mean was 10.2 ($n$ = 60), and the relative standard deviation was 1.14%.

*Sensitivity*—The sensitivity of the procedure was determined by assaying a series of cephradine standard solutions [10–1000 μg/ml (potency of 9–900 μg/ml)] prepared in 0.1 *M* phosphate buffer, pH 7.0. Figure 3 shows that the relationship between iodine uptake and potency was linear between 25 and 720 μg/ml. Because of low iodine uptake and concomitantly greater thiosulfate titration values, the method was unworkable at cephradine potencies less than 25 μg/ml.

In addition to the daily determination of iodine titer, the linearity of the relationship between iodine uptake and potency of cephradine also obviated the need to construct a complete standard curve each day that the assay was performed. It was concluded that use of a single standard solution of cephradine, with a potency between 25 and 720 μg/ml, would be sufficient to determine the iodine titer in routine analyses.

*Effect of 7-Aminodeacetoxycephalosporanic Acid*—Since 7-aminodeacetoxycephalosporanic acid is an intermediate in the production of cephradine and has a typical β-lactam ring structure, its possible interference in the iodometric assay was investigated. A standard solution of cephradine in 0.1 *M* phosphate buffer, pH 7.0, with a concentration of 503 μg/ml was assayed in the presence of 7-aminodeacetoxycephalosporanic acid representing 1–20% of the weight of cephradine in the reaction mixture. Reaction mixtures and controls were assayed as described under *Methods;* the results (Table II) showed that 7-aminodeacetoxycephalosporanic acid did not interfere in the assay; *i.e.*, there was no significant change in the concentration obtained when cephradine was assayed in the presence of its precursor.

In another experiment, iodine uptake by 7-aminodeacetoxycephalosporanic acid after treatment with cephradinase was compared with that after treatment of the compound with alkali, as described for cephradine under *Methods.* The results (Table III) showed that almost no iodine was taken up by 7-aminodeacetoxycephalosporanic acid after treatment with the enzyme, whereas substantial amounts were consumed after treatment with alkali. From these results, it was concluded that 7-aminodeacetoxycephalosporanic acid was not readily hydrolyzed by cephradinase and that contaminating amounts of the precursor would not affect the results of an iodometric assay of cephradine that used the enzyme for hydrolysis of the β-lactam ring. In contrast, for the method using alkaline hydrolysis, contaminating amounts of 7-aminodeacetoxycephalosporanic acid would be expected to give erroneously high concentrations of cephradine.

*Comparison with Bioassay and Alkali Iodometric Method*—The iodometric method using enzymatic hydrolysis was compared with a turbidimetric bioassay, in which *Streptococcus faecalis* (ATCC 10541) was the test organism (2), and with the iodometric method involving alkaline hydrolysis. Solutions (~500 μg/ml) of six cephradine powders in 0.1 *M* phosphate buffer, pH 7.0, were prepared for the two iodometric assays; solutions (~250 μg/ml) of cephradine in 0.1 *M* phosphate buffer, pH 3.5, were prepared for the bioassay. The solutions were assayed by each method, on 2 consecutive days, fresh solutions and standards being prepared for each day of assay.

The potencies obtained for the cephradine powders (some of which had been stored at high temperatures for several years) used in this

**Table I—Precision of the Enzyme Iodometric Method for Cephradine**[a]

| Operator | Potency Range, μg/ml | Mean Potency, μg/ml | *SD* of Mean ($n$ = 60) |
|---|---|---|---|
| 1 | 912–867 | 893 | 10.2 |
| 2 | 902–887 | 895 | |

[a] Assays were carried out by the procedure described under *Methods.* Relative standard deviation = 1.14%. Three samples were analyzed 30 times.

**Table II—Effect of 7-Aminodeacetoxycephalosporanic Acid on the Potency of Cephradine as Determined by Enzyme Iodometric Assay**[a]

| Amount of 7-Aminodeacetoxycephalosporanic Acid Added to Cephradine, % (w/w) | Observed Potency, μg/mg | Change in Observed Potency, % |
|---|---|---|
| 0.0 | 891.2 | — |
| 1.0 | 891.2 | 0 |
| 2.0 | 891.1 | 0 |
| 5.0 | 891.2 | 0 |
| 10.0 | 895.6 | +0.5 |
| 20.0 | 895.8 | +0.5 |

[a] Results are the means of duplicate determinations. Assays were carried out as described under *Methods.* Reaction mixtures were prepared to contain 4.0 ml of a standard solution of cephradine (concentration of 503 μg/ml) and 1.0 ml of a solution of 7-aminodeacetoxycephalosporanic acid containing 1–20% 7-aminodeacetoxycephalosporanic acid by weight of the amount of cephradine in the reaction mixture.

Table III—Iodine Uptake by 7-Aminodeacetoxycephalosporanic Acid and Cephradine after Treatment with Alkali or Cephradinase for 30 min[a]

| Compound in Solution | Iodine Uptake[b] After Alkali[c] Treatment | Iodine Uptake[b] After Cephradinase[d] Treatment |
|---|---|---|
| Cephradine (530 μg/ml) | 1.64 | 2.19 |
| 7-Aminodeacetoxy-cephalosporanic acid (510 μg/ml) | 1.76 | 0.01 |
| 7-Aminodeacetoxy-cephalosporanic acid (102 μg/ml) | 1.34 | 0.01 |
| 7-Aminodeacetoxy-cephalosporanic acid (5.1 μg/ml) | 0 | 0 |

[a] Results are the means of duplicate determinations. [b] Iodine uptake = milliliters per milligram of 7-aminodeacetoxycephalosporanic acid or cephradine. [c] Iodometric assays involving alkaline hydrolysis were carried out as described under *Methods*. [d] Iodometric assays involving enzymatic hydrolysis were carried out as described under *Methods*.

experiment are given in Table IV. These results showed that the potencies determined by the iodometric method involving cephradinase hydrolysis agreed well with those obtained by bioassay, except when loss of bioactivity exceeded 50%. In contrast, the potencies determined by the alkali iodometric assay always showed poor agreement with the results of the bioassay.

**Spectrophotometric Assay**—Standard curves for the determination of cephradine potencies by spectrophotometric analysis after enzymatic hydrolysis of the β-lactam ring were prepared from the difference between absorbances obtained after reaction at room temperature of the cephradine standards with cephradinase and with cephradinase boiled for 30 min. The standard curves obtained were always linear, passing through the origin, and obeyed Beer's law for potencies of cephradine between 1 and 300 μg/ml.

*Precision and Accuracy*—The precision and accuracy were determined by assaying standard solutions of pure cephradine dihydrate of the same concentration (40 μg/ml, potency of 36 μg/ml) on 3 consecutive days. For a total of 60 determinations carried out by one operator over the 3-day period ($n$ = 20/day), the recovery was 99.2%, the standard deviation of the mean was 1.52 ($n$ = 60), and the relative standard deviation was 4.28%.

*Sensitivity*—With 1-cm light path length cells, the assay could be used to measure cephradine potencies as low as 1 μg/ml and as high as 300 μg/ml. This range possibly could be extended by using cells of different path lengths.

Table IV—Comparative Potencies of Cephradine in Bulk Powders[a]

| Sample | Cephradine Potency, μg/mg: Cephradinase Iodometric Assay[b] | Alkali Iodometric Assay[c] | Bioassay[d] |
|---|---|---|---|
| NN054ND | 813 | 979 | 812 |
| NN059ND | 843 | 991 | 854 |
| NN061ND | 846 | 980 | 778 |
| NN054ND (large bottle) | 578 | 744 | 578 |
| NN059ND (large bottle) | 466 | 643 | 424 |
| NN061ND (large bottle) | 464 | 639 | 405 |

[a] Results are the means of duplicate determinations on 2 consecutive days. Unless otherwise noted, the powders were stored at 50° in standard bottles for more than 2 years. [b] Iodometric assays involving enzymatic hydrolysis were carried out as described under *Methods*. [c] Iodometric assays involving alkaline hydrolysis were carried out as described under *Methods*. [d] Bioassays were carried out by the turbidimetric method (2).

Table V—Potency of Cephradine Formulations Determined by Spectrophotometric Assay and Bioassay[a]

| Formulation | Spectrophotometric Assay[b] | Bioassay[c] |
|---|---|---|
| Capsules | 480.3 | 482.0 |
| Oral suspensions | 39.8 | 38.7 |
| Powders for reconstitution for injection | 300.3 | 288.7 |

[a] Results are the means of duplicate determinations on 10 samples of each formulation. Cephradine potency is given as milligrams per capsule, milligrams per gram (oral suspension), or milligrams per vial (powder). [b] Spectrophotometric assays were carried out as described under *Methods*. [c] Bioassays were carried out by the turbidimetric method (2).

*Comparison with Bioassay*—Since the spectrophotometric method was intended for use in routine assays of cephradine in powders and other formulations, it was compared with the more commonly used bioassay in a series of experiments. The bioassay routinely used in these laboratories was a turbidimetric method (2), employing *S. faecalis* as the test organism. Accordingly, a series of capsules containing cephradine was analyzed in duplicate by both methods.

The results (Table V) showed that there was good agreement between the results obtained for this formulation. With other cephradine formulations, however, the spectrophotometric method gave consistently greater potency values compared with the bioassay (Table V). A similar discrepancy was also observed when the method was used to determine the potencies of cephradine powders stored for long periods at high temperatures. Table VI shows that, although the spectrophotometric method did indicate a loss of potency in certain samples, the potency loss was not as great as that indicated by the bioassay.

## DISCUSSION

An iodometric assay of cephradine that uses the β-lactamase, cephradinase, in place of alkali for hydrolysis of the β-lactam ring was developed as a modification of the original iodometric method described by Alicino (3, 9). The procedure is relatively straightforward, accurate, and precise and gives reproducible results. It also is more specific than the original iodometric method that used alkaline hydrolysis[7], probably due to the more specific hydrolysis of the β-lactam ring by the enzyme.

However, the method cannot differentiate cephradine from other cephalosporins, such as cephalexin, which are substrates of the enzyme. These compounds can be readily assayed by exactly the same procedure as that described for use with cephradine. For each new cephalosporin, however, new reaction conditions (*e.g.*, time of incubation with the enzyme and time of iodination) have to be established. Interference in the method by the penicillins is unlikely, because these compounds are hydrolyzed by the enzyme only very slowly.

The method proved to be as accurate as the bioassay in determining the potencies of cephradine bulk formulations used in long-term stability studies, but it has the advantage of being more precise and

Table VI—Potencies of Cephradine Powders Subjected to Long-Term Storage as Determined by Spectrophotometric Assay and Bioassay[a]

| Sample Number and Storage Conditions | Cephradine Potency, μg/mg: Spectrophotometric Assay[b] | Bioassay[c] |
|---|---|---|
| 59, 5° | 978 | 867 |
| 59, 50° | 915 | 774 |
| 59 Bulk, 50° | 537 | 484 |
| 61, 5° | 946 | 903 |
| 61, 50° | 910 | 791 |
| 61 Bulk, 50° | 581 | 500 |

[a] Results are the means of duplicate determinations. [b] Spectrophotometric assays were carried out as described under *Methods*. [c] Bioassays were carried out by the turbidimetric method (2).

more rapid to perform than the bioassay. Although the procedure can be used to assay cephradine with a potency as low as 25 μg/ml, it lacks the sensitivity of the bioassay.

Since iodine was added to the reaction mixtures only after hydrolysis of the cephradine by cephradinase had been completed, a possible decrease in activity of the enzyme resulting from contact with the iodine was unimportant. The enzyme itself did not take up any iodine during the iodination procedure. The reaction was carried out at room temperature for the sake of simplicity but, from the results reported by others (14, 15), hydrolysis of the β-lactam ring at higher temperatures would be expected to give a more rapid assay and could lead to the development of an automated procedure similar to that reported by Lindstrom and Nordstrom (16).

The spectrophotometric method described was developed as a modification of certain methods used to assay β-lactamase activity (14, 17). It is similar to the procedures described for penicillin (18), for cephalosporin C (10), and for ampicillin (19). The procedure appears to be unique, however, in quantitating the amount of cephradine by measuring the difference between the absorption at 260 nm of enzymically hydrolyzed and nonhydrolyzed samples of the antibiotic. Use of a double-beam instrument and comparison of a hydrolzed sample with its nonhydrolyzed control yielded a direct reading of the amount of cephradine present. Also, at the wavelength used, this method of reading the absorbances eliminated the effects of absorbance of nonantibiotic material and enzyme.

The method proved to be quick, simple, and precise and gave reliable quantitative results. It has the advantage of requiring only small amounts of cephradine, and the range of the assay is limited only by the capabilities of the spectrophotometer. The assay is specific for cephalosporins, since the other major group of β-lactam-containing antibiotics, the penicillins, are poor substrates of the enzyme[1] and do not exhibit absorption maxima at 260 nm (8). Like the enzyme iodometric assay, this procedure cannot be expected to distinguish between cephradine and other cephalosporins that also serve as substrates for the enzyme[1].

Although the results obtained by this procedure in the assay of cephradine in dry-filled capsules compared favorably with those of the bioassay, high results obtained in the assay of other formulations made the method less accurate than the bioassay. The procedure also proved unsatisfactory for determining potencies of cephradine powders stored for long periods. Although the spectrophotometric assay showed decreases in potency of these powders that had been stored at high temperatures for 2 years, the decreases were not as great as those shown by the bioassay. The procedure also gave higher results than the bioassay for powders stored at 5°.

This discrepancy was probably attributable to the formation of nonbioactive or less bioactive products containing a β-lactam ring from cephradine during its storage. As noted for other antibiotics (10), the difference between the results of the chemical (spectrophotometric) assay and the bioassay of cephradine increased significantly and progressively as degradation of the drug proceeded.

Of the two chemical methods described, the iodometric appears to be the more suitable for determining the potency and stability of cephradine. The spectrophotometric method, which is more rapid but less accurate than the iodometric assay, should be the method of choice when a rapid determination of relative change in potency is desired.

## REFERENCES


(1) R. J. Simmons, in "Analytical Microbiology," vol. 2, F. Kavanagh, Ed., Academic, New York, N.Y., 1972, pp. 193–205.

(2) D. Pitkin, P. Actor, W. Holl, A. Post, and J. A. Weisbach, *Antimicrob. Ag. Chemother.*, **5**, 223(1974).

(3) J. F. Alicino, *Ind. Chem. Eng., Anal. Ed.*, **18**, 619(1946).

(4) J. H. Ford, *Anal. Chem.*, **19**, 1004(1947).

(5) R. J. Henry and R. D. Housewright, *J. Biol. Chem.*, **167**, 559(1947).

(6) W. S. Wise and G. H. Twigg, *Analyst*, **75**, 106(1950).

(7) C. H. O'Callaghan, P. W. Muggleton, and G. W. Ross, *Antimicrob. Ag. Chemother.*, **1968**, 57.

(8) A. Samuni, *Anal. Biochem.*, **63**, 17(1975).

(9) J. F. Alicino, *Anal. Chem.*, **33**, 648(1961).

(10) L. P. Marelli, in "Cephalosporins and Penicillins, Chemistry and Biology," E. H. Flynn, Ed., Academic, New York, N.Y., 1972, pp. 609–635.

(11) T. D. Hennessey and M. H. Richmond, *Biochem. J.*, **109**, 469(1968).

(12) G. Gomori, in "Methods in Enzymology," vol. 1, S. P. Colowick and N. O. Kaplan, Eds., Academic, New York, N.Y., 1955, pp. 138–146.

(13) "Code of Federal Regulations," Title 21, Section 141 a.35(b), June 1, 1974.

(14) G. W. Ross, K. V. Chanter, A. M. Harris, S. M. Kirby, M. J. Marshall, and C. H. O'Callaghan, *Anal. Biochem.*, **54**, 9(1973).

(15) C. J. Perret, *Nature (London)*, **174**, 1012(1954).

(16) E. B. Lindstrom and K. Nordstrom, *Antimicrob. Ag. Chemother.*, **1**, 100(1972).

(17) L. D. Sabath, M. Jago, and E. P. Abraham, *Biochem. J.*, **96**, 739(1965).

(18) S. G. Waley, *ibid.*, **139**, 789(1974).

(19) J. A. T. Jansson, *Biochim. Biophys. Acta*, **99**, 171(1965).


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