the accelerator in question may be bound to varying degrees to the micelles and other components of the synthetic bile. Thus, meaningful structure–activity relationships can only be considered when the "free" concentration, or the thermodynamic activity, of the accelerator is known. Further progress in this area depends on conducting studies in which the "intrinsic" activities of these agents can be determined.

A method based on the dialysis rate principle for determining the free accelerator concentration in simulated bile is being developed and appears feasible (14). It appears, for example, that the actual (or the intrinsic) difference between n-hexylamine and n-octylamine in the cholic acid–lecithin media is closer to a factor of 10 or 15 than to the marginal difference seen in Table I. As such data become available, one may begin to understand the action of these accelerators at the molecular level.

Of course, the search for suitable adjuvants for gallstone dissolution in humans via the oral route will require additional studies on the absorption, biliary excretion, metabolism, and toxicity of these compounds. While this fact may appear to make this approach impractical or very difficult, one needs only to note that VIb, 20,25-diazacholesterol, an established hypocholesterolemic agent, is orally absorbed in humans (15, 16) and in other animal species. Studies also showed (17) that significant percentages of high molecular weight quaternary ammonium cations such as dibenzyldimethylammonium, tribenzylmethylammonium, and benzomethamine are excreted in quantity in rat, rabbit, and human bile when given intravenously. Therefore, compounds such as the steroidal amines and the bile acid derivatives containing the amine nitrogen or the quaternary nitrogen functional groups may offer promise for this approach.

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ACKNOWLEDGMENTS AND ADDRESSES

Spectrophotometric Determination of Cephapirin, a Cephalosporin Antibacterial

## J. E. BODNAR, W. G. EVANS, and D. L. MAYS ˣ

Abstract □ A simple and specific method for the quantitative determination of cephapirin, a cephalosporin antibacterial, in finished bulk and dosage forms is reported. The method is based on reproducible degradation under controlled conditions to an unidentified species, which is measured spectrophotometrically at 375 nm. The procedure can be performed manually on a short series of samples in about 15 min or can be automated for large runs. Precursors and related substances show minimal interference. The coefficient of variation of the automated system is about 1% within days and 1.3% among days.

Keyphrases □ Cephapirin—spectrophotometric analysis, bulk drug and pharmaceutical formulations □ Spectrophotometry—analysis, cephapirin, bulk drug and pharmaceutical formulations □ Antibacterials—cephapirin, spectrophotometric analysis, bulk drug and pharmaceutical formulations

Cephapirin sodium (I), a new cephalosporin antibacterial for parenteral use, has the advantage of being less painful and better tolerated than cephalothin (1). The two methods of analysis officially approved for certification are the microbiological agar–plate diffusion method and the colorimetric hydroxylamine method (2). However, this paper reports a more rapid and selective spectrophotometric method for convenient and accurate product control of finished bulk and dosage forms. The method has been automated to accommodate large numbers of stability samples. Products arising through hydrolysis of the acetyl function show little or no interference. The method is selective for only a few cephalosporins.

Manual and automated procedures, the effects of several operating conditions, and validation of the method are presented.
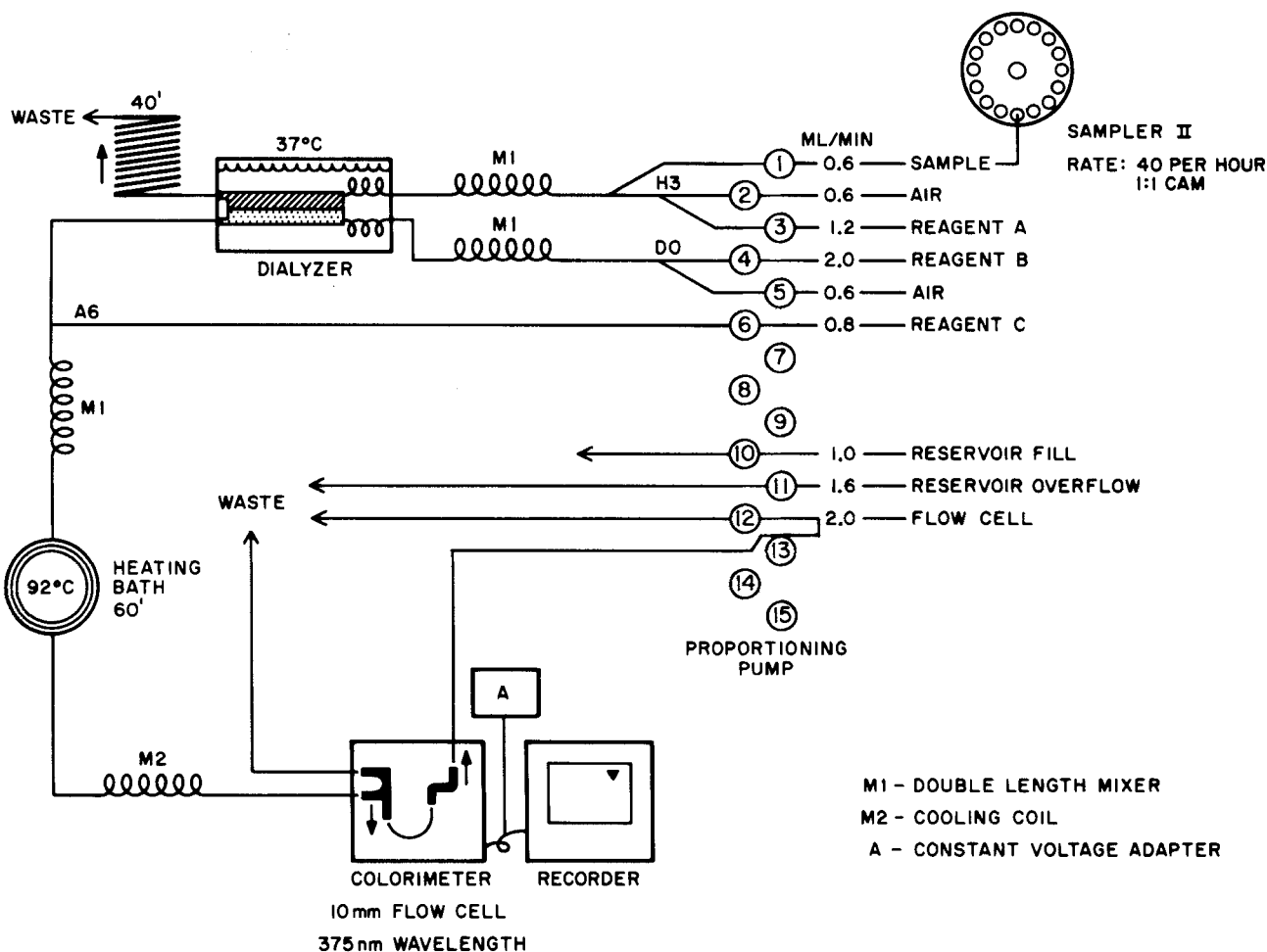

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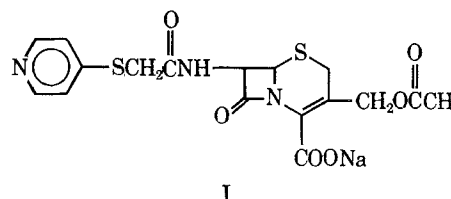
Figure 1—*Flow diagram of automated spectrophotometric assay for cephapirin.*

EXPERIMENTAL

Instrumentation—A standard pH meter[1] and two spectrophotometers[2] were used for developing the manual method. The automated system consisted of a continuous-flow analyzer[3] assembled as shown in Fig. 1. The spectrophotometer[4] output was fed into the recorder[3] *via* a special adaptor for single-voltage input (3). A proportional temperature controller[5] was used to maintain the heating bath temperature.

Reagents—*Manual Method*—The sample diluent was prepared by dissolving 51 g of potassium biphthalate[6] and 0.9 g of imidazole[7] in 800 ml of water, adjusting the pH to 6.50 ± 0.02 with 10 N NaOH, and diluting to 1 liter with water.

Automated Method—The sample diluent was prepared as for the manual method but without the imidazole. This reagent, with the addition of 5 drops/liter of polysorbate 20[8], was also used for the continuous-flow analyzer (Reagent A). The dialyzer recipient (Reagent B) was 0.2 M NaCl with 5 drops/liter of polysorbate 20. Reagent C was prepared by dissolving 23 g of potassium biphthalate and 1.0 g of imidazole in 450 ml of water, adjusting the pH to 6.50 ± 0.02 with 10 N NaOH, and diluting to 500 ml with water.

Procedure—*Manual Determination*—Dissolve 50 mg of cephapirin sodium reference standard in 100 ml of sample diluent. Dilute 5-, 10-, 15-, 20-, and 25-ml aliquots to 50 ml with the diluent. Dissolve samples in diluent to contain 0.1–0.2 mg of cephapirin/ml. Transfer 4 ml of each dilution to separate 25-ml volumetric flasks. Stopper the flasks and heat
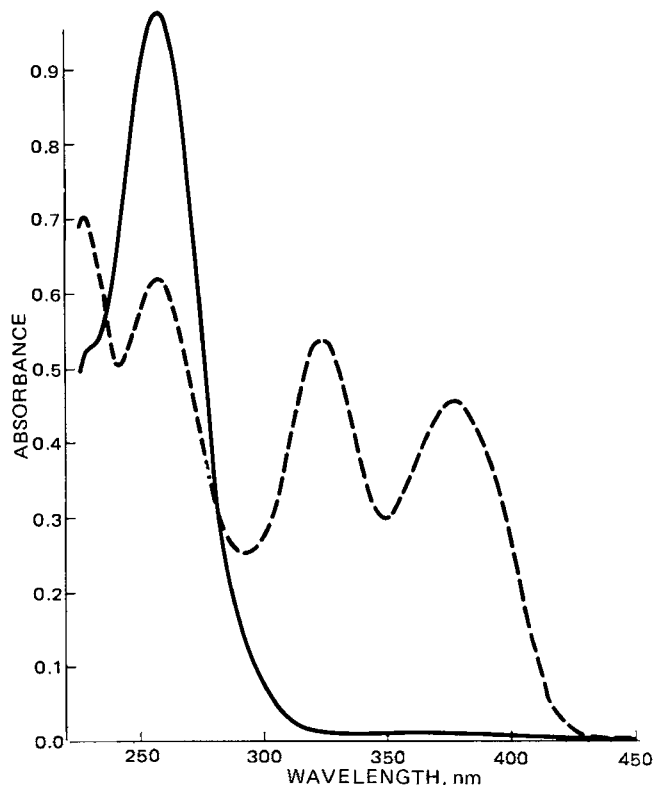
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[1] Radiometer model 26.
[2] Beckman DU and Cary model 14.
[3] Technicon Autoanalyzer modules as follows: Sampler II with a 40/hr 1:1 cam, Pump II, a dialyzer (37°), a heating bath (92°) containing 6.1- and 12.2-m (20- and 40-ft) glass holding coils, and a single-pen recorder.
[4] Turner model 350.
[5] RFL Industries model 7-115 with Sensor 27686-3.
[6] Matheson, Coleman & Bell.
[7] Eastman Chemical Co.
[8] Tween 20, Atlas Products.

Figure 2—*Absorption spectra of cephapirin in pH 6.5 phosphate buffer unheated (—) and heated for 9 min at 100° (- -).*
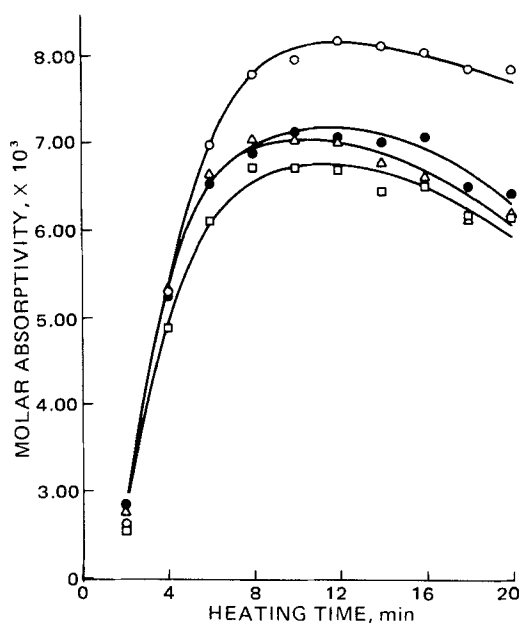
Figure 3—*Rate of formation of 375-nm chromophore as a function of heating time (100°) in several buffers. Key: □, 0.25 M pH 6.5 phthalate; △, 0.1 M pH 6.5 phosphate; ●, 0.1 M pH 6.5 acetate; and ○, 0.25 M pH 6.5 phthalate plus 0.013 M imidazole.*

in a boiling water bath for exactly 9 min.

Immediately after heating, cool the flasks in an ice water bath for about 1 min. Then allow the flasks to equilibrate to room temperature, and dilute them to volume with diluent. Prepare the blanks similarly, but do not heat or cool. Determine the absorbance of each standard at 375 nm *versus* the respective blank. Prepare a standard curve of absorbance *versus* concentration. Calculate the cephapirin content of the samples from the standard curve.

Automated Determination—Dissolve 100 mg of cephapirin sodium reference standard in 100 ml of sample diluent. Dilute 5-, 10-, 15-, 20-, and 25-ml aliquots to 25 ml with sample diluent. Prepare samples to contain about 0.5 mg of drug/ml of sample diluent. Fill the sample cups with standard and sample solutions, applying a set of standards at both the beginning and end of each series of samples. Sample blanks are not required but could be obtained by lowering the heating bath temperature to 25°. Obtain the absorbance from the peak maxima of standards and samples. Prepare a working curve of absorbance *versus* concentration, and calculate the cephapirin content of samples from the working curve.

RESULTS AND DISCUSSION

Optimum Conditions—The UV absorption spectra of heated and unheated cephapirin in pH 6.5 phosphate buffer are shown in Fig. 2. The

Table I—Within-Day Reproducibility of an Automated Spectrophotometric Determination of Cephapirin

| Sample | Percent Cephapirin in Cephapirin Sodium | | | | Coefficient of Variation, % |
	Run 1	Run 2	Run 3	Mean	
			Day 1		
A	93.4	94.3		93.8	0.66
B	82.8	83.6		83.2	0.68
C	85.2	83.0		84.1	1.85
D	89.5	88.0		88.8	1.20
E	88.4	86.5		87.4	1.53
F	91.1	90.5		90.8	0.46
Pooled standard error = 1.28%					
			Day 2		
A	94.3	93.7	95.7	94.6	1.59
B	84.1	83.2	84.2	83.8	0.65
C	80.8	81.4	81.2	81.1	0.38
D	91.1	89.2	91.1	90.5	1.21
E	90.7	91.1	90.2	90.7	0.50
F	91.9	92.1	91.3	91.8	0.46
Pooled standard error = 0.92%					

Table II—Across-Day Reproducibility of an Automated Spectrophotometric Determination of Cephapirin

| Sample | Percent Cephapirin in Cephapirin Sodium | | | | | | Coefficient of Variation, % |
	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	
G	94.1	95.2	94.3	93.1	94.2	94.2	0.8
H	94.1	94.7	95.5	93.3	94.9	94.5	0.9
I	93.8	94.8	95.1	92.7	92.7	93.8	1.2
Pooled standard error = 0.98%							

treated samples showed partial loss of the main peak at 258 nm and generation of new peaks at 320 and 375 nm. The peak at 375 nm was chosen for analytical purposes, since interferences generally occurred less frequently toward the visible region. The unheated sample made a suitable blank. When heated at 100°, the chromophore increased rapidly, reaching a maximum in about 9 min, and then decreased slowly (Fig. 3). The absorbance remained constant for at least 1 hr after cooling.

A plot of absorbance at 375 nm *versus* concentration was linear and passed through the origin, obeying Beer's law over a fivefold concentration range (8–40 μg/ml in the final dilution for the manual method and 200–1000 μg/ml in the sample cup for the automated method). The limit of detection was about 2 μg/ml for the manual method and about 50 μg/ml for the automated procedure.

The chromophore intensity was pH dependent, with the maximum response observed at pH 6.5 (Fig. 4). Phosphate, acetate, or phthalate buffers can be used (Fig. 3). Other buffers (borate, citrate, glycine, and tromethamine), especially those containing chloride ion, are not suitable. Sample absorbance was increased in the presence of imidazole but was relatively independent of imidazole concentration over a broad range (Figs. 3 and 5).

Reproducibility and Accuracy—The pooled standard error among automated runs within 1 day on an assortment of crude samples was calculated from replicate runs made on 2 separate days (Table I). In both cases, the within-day standard error was less than 1.5%. The reproducibility of results across days is shown in Table II. A comparison of results obtained between days on good quality samples yielded a pooled standard error of less than 1%.

Results of a series of samples analyzed by both the automated spectrophotometric and the biological turbidimetric methods are shown in Table III. Results of the proposed procedure agreed within 1% overall with those of the standard biological method. The average absolute error was 1.7%.

Specificity—There appeared to be little or no interference from 7-aminocephalosporanic acid or the desacetyl derivative of cephapirin and only slight interference from the lactone of the desacetyl derivative, all potential impurities in cephapirin (Table IV). Cephapirin treated for 15 min with cephalosporinase[9] yielded no measurable 375-nm chromophore upon heating, demonstrating that cephapirin degradation products arising *via* β-lactam cleavage did not interfere.

Several commercial cephalosporins and intermediates in cephapirin production were screened for applicability of the method. In addition
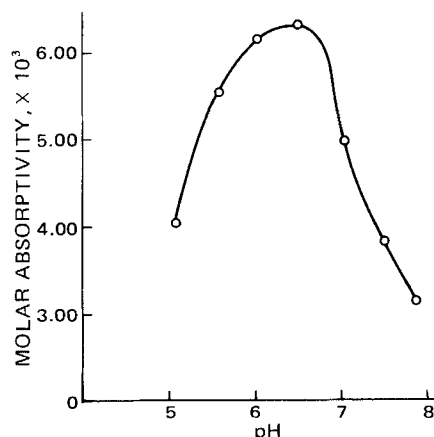

Figure 4—*Extent of formation of 375-nm chromophore as a function of pH in 0.25 M phthalate buffer without imidazole (heated for 9 min at 100°).*

[9] Obtained from *Enterobacter cloacae*, P 99.

Table III—Comparison of Automated Spectrophotometric and Biological Turbidimetric Methods of Determination of Cephapirin (Percent) in Cephapirin Sodium

Sample	Chemical Method[a]	Biological Method[b]
1	94.0	90.5
2	92.8	91.5
3	90.5	92.5
4	92.5	93.5
5	90.3	93.5
6	93.6	93.5
7	92.7	90.3
8	93.9	92.7
9	91.9	93.0
10	94.2	91.0
11	94.3	92.0
12	94.2	92.0
13	94.2	91.5
14	94.5	92.0
15	93.8	93.5
16	94.2	94.0
17	94.2	95.0
18	93.2	93.5
19	92.6	94.0
Average	93.2	92.5

Average absolute deviation = 1.7%
Overall deviation = 0.7%

[a] Each result is the mean of four determinations. [b] Each result is the mean of individual determinations made on 2 or 3 separate days.

Table IV—Percent Recovery of Assayed Cephapirin Content of Cephapirin Sodium after Spiking with Potential Impurities

Impurity Added	Run	Proportion of Impurity Added 1%	5%	10%	Grand Mean, %
7-Aminocephalosporanic acid	1	98.8	99.6	100.4	
	2	99.6	100.4	100.4	
	3	100.9	100.9	101.7	
	Mean	99.8	100.3	100.8	100.3
Desacetyl derivative of cephapirin	1	99.6	98.0	99.6	
	2	98.1	98.1	98.1	
	3	100.9	100.9	102.4	
	Mean	99.5	99.0	100.0	99.5
Lactone of desacetyl derivative	1	100.4	101.2	102.7	
	2	100.4	101.2	103.5	
	Mean	100.4	101.2	103.1	101.6

to cephapirin, cephalothin sodium and cephalosporin D sodium[10] can be determined by this method. Cephalosporins that do not respond to the test include cephaloridine, cefazolin sodium, cephalexin, and cephalosporin C sodium.

Mechanism of Reaction—Consideration of the structures of the cephalosporins that respond to the test indicates that an easily displaced 3-methyl substituent is required for chromophore formation and that a primary amine in the 7-position substituent inhibits the conversion. The heated solution appeared to contain a mixture of products, since some of the 258-nm absorption band remained. The species absorbing at 375 nm has not been isolated. When using TLC or column chromatography, the concentration of the test solution was insufficient. At a substantially greater concentration, the conversion was very poor and essentially cephapirin was recovered. Upon lyophilization, the 375-nm absorption was lost, indicating either further degradation or loss by volatility.

Upon heating under controlled conditions, penicillins rearrange to penicillenic acids which have UV absorption around 320 nm. These penicillenic acids are stabilized by copper or mercury salts and catalyzed by imidazole to give much higher conversion yields (4, 5). Addition of copper sulfate or mercuric chloride to the cephapirin test solution drastically diminished the 375-nm absorbance, indicating that a penicillenic acid type of rearrangement was not involved.

If external or internal nucleophilic displacement of the acetoxy group is involved, then addition of reagents known to displace this group might
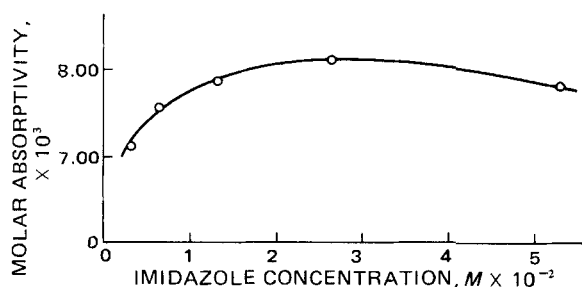
be expected to increase or decrease the reaction yield. Various heterocyclic tertiary amines (6, 7) including niacinamide (8) displace the acetoxy group. Addition of niacinamide had no effect on chromophore development, which may suggest that any displacement of the acetoxy group must occur from internal expulsion of that group as a result of an internal rearrangement.

Imidazole is known to catalyze hydroxylaminolysis of the β-lactam of penicillins (9). The addition of imidazole to this reaction showed some increase in yield of the 375-nm absorbing species, and the amount of yield increase was only slightly dependent on imidazole concentration over a broad range. This finding might suggest that the reaction is initiated through the β-lactam of cephapirin and is catalyzed by imidazole. Some molecular rearrangement occurs, giving perhaps a number of products, one or more of which absorb at 375 nm. The apparent necessity of the acetoxy group in the molecule may simply correlate with those cephalosporins having less stable β-lactams. An optimum pH of near neutral is consistent, since simple lactam hydrolysis with further rapid degradation may compete with the observed rearrangement at higher pH values.

These suggestions, however, are quite speculative; definite conclusions must await more evidence.

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Figure 5—*Molar absorptivity at 375 nm in 0.25 M phthalate buffer as a function of imidazole concentration (heated for 9 min at 100°).*

[10] Cephalosporin D, the *N*-isobutyloxycarbonyl derivative of cephalosporin C, is an intermediate in the production of 7-aminocephalosporanic acid.