on the 5th and 6th days of infection (when the parasitemia was >0.01%) were trypanocidal to such an extent that no parasites were detected over 90 days. The mice were tested every 15 days (Fig. 1). The other drugs, including the parent drugs (I and II), were less effective; they lowered parasitemias significantly but did not eliminate the parasites totally. Mice administered congocidine (I) or IV (40 mg/kg) three or more times died either from high parasitemia or from the toxic effect of the drug. Compound II was less toxic but also less effective against the parasite as compared with I. Six doses of II (40 mg/kg) did not affect the growth of the parasites, while five doses of 80 mg/kg inhibited the growth only partially.

*In vitro* studies indicated that III was also the most effective compound against *L. tropica* amastigotes in $C_3H$ mouse mononuclear phagocytes (Fig. 2). In cultures treated with 10 μg of III/ml, the parasite survival index indicated 50% survival on the 2nd day and 20% by the 4th day. Compounds IV (25 μg/ml) and II (100 μg/ml) also reduced growth but were not as effective as III. Cytotoxicity to host cells was observed at 25, 50, 50, and 200 μg/ml of I, III, IV, and II, respectively.

The doses used were the minimal effective nontoxic ones. Thus, no inhibitory effects on the growth of *L. tropica* were seen when II and IV were given in a concentration of <100 and 25 μg/ml, respectively. The monopyrrole analog of congocidine (VI) had the same effect against *T. congolense* as the parent drug. Its position isomer (VII) was completely inactive.

## DISCUSSION

In two antiparasitic tests, the tripyrrole derivative of congocidine (III) was more potent and less toxic than congocidine and more potent than its analog (IV). Although less toxic than congocidine, IV demonstrated the same inhibitory activity as the parent drug in the two systems tested. These results showed that there are considerable structural requirements in the congocidine series for their antiparasitic activity.

It was reported previously (15) that the tripeptide (IV) is more active in some antiviral tests than the dipeptide congocidine (I). However, this modification alone did not improve the antiparasitic activity, although it improved the therapeutic index. Surprisingly, the addition of a methyl group to the β-aminopropionamidine moiety in IV, leading to III, increased the antiparasitic activity and further improved the therapeutic index. The fact that distamycin A was totally inactive in the two antiparasitic tests demonstrated that the guanidinoacetyl moiety in the congocidine molecule cannot be replaced by a formamido moiety, contrary to observations in the antiviral field.

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# High-Performance Liquid Chromatographic Analysis of Carbenicillin and Its Degradation Products


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Received November 19, 1980, from the *Penicillin Recovery Department, Pfizer Inc., Groton, CT 06340.* Accepted for publication December 22, 1980.



**Abstract** □ An ion-pair reversed-phase high-performance liquid chromatographic system is used to separate carbenicillin from carbenicillin penicilloic acid, carbenicillin penilloic acid, penicillin G, penicillin G penicilloic acid, and penicillin G penilloic acid. Conditions are provided for resolution of the carbenicillin diastereomer pair and for elution as a single peak, facilitating quantitation.



**Keyphrases** □ Carbenicillin—degradation products, high-performance liquid chromatographic analysis, mobile phase modifications □ High-performance liquid chromatography—analysis, carbenicillin, resolution of diastereomer pair or single-peak elution by mobile phase modifications □ Antibiotics—carbenicillin, high-performance liquid chromatographic analysis, mobile phase modifications □ Degradation—carbenicillin, high-performance liquid chromatographic analysis


Carbenicillin, a broad-spectrum semisynthetic penicillin-type antibiotic, is important because of its antipseudomonal and antiproteus effect. Most classical analytical methods available for penicillin purity or potency determinations are applicable to carbenicillin; the iodometric and hydroxylamine methods and the microbiological agar diffusion assay are the most widely used. The latter method is the one specified by the Antibiotic Regulations of the Food and Drug Administration (1). Carbenicillin monosodium monohydrate is used as a reference standard for this assay (2).

## BACKGROUND

Recent literature (3–6) indicated that high-performance liquid chromatography (HPLC), especially reversed-phase or ion-pair reversed-phase HPLC on microparticulate (≤10 μm) bonded packing materials, is amenable to the analysis of penicillins. In many penicillin analyses,




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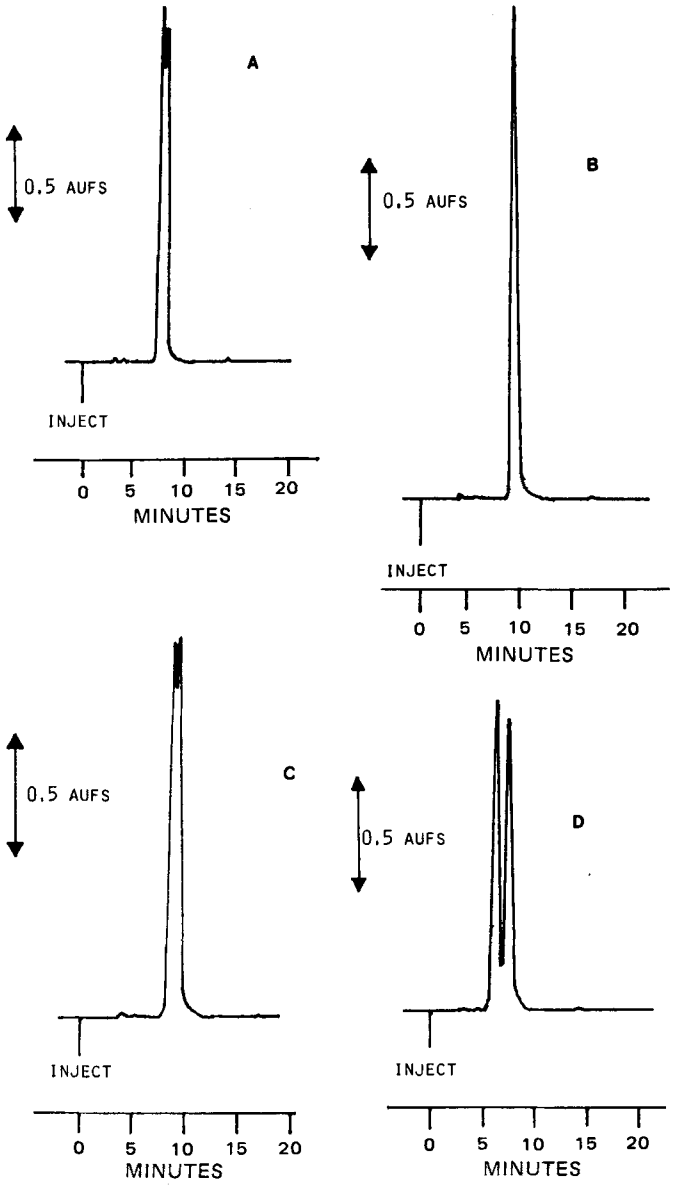


**Figure 1**—*Chromatograms of carbenicillin (0.850 mg/ml) with a mobile phase of 37% methanol at 1.2 ml/min and the detector wavelength at 220 nm. Key (pH of mobile phase): A, 3.00; B, 3.35; C, 3.70; and D, 4.35.*

HPLC procedures are the methods of choice because of demonstrated precision, accuracy, speed, and ability to provide ancillary information relative to the nature and identity of purity-depressing substances such as reaction by-products or natural degradation products present in the sample. In addition, the analyses often can be accomplished when the drug is part of a complex mixture such as a biological fluid or blend formulation.

Carbenicillin is a somewhat unique semisynthetic penicillin in that the normal synthesis routes produce a diastereomer mixture. This mixture complicates quantitative analysis by HPLC since reversed-phase microparticulate column packings generally are selected for their ability to separate the components of a mixture. Thus, the same criterion used to select HPLC as an analytical method for carbenicillin can negate its quantitation since the carbenicillin diastereomers tend to elute as two peaks, except when the chromatographic conditions are closely controlled. This tendency is especially true when methanolic mobile phases and high efficiency columns are used.

This report describes the mobile phase modifications used with a 5-$\mu$m octadecylsilyl-bonded silica-based column to provide either separation of the carbenicillin diastereomers or elution as one peak, depending on the desires of the analyst. In addition, separation of carbenicillin from some of its possible degradation products is demonstrated.

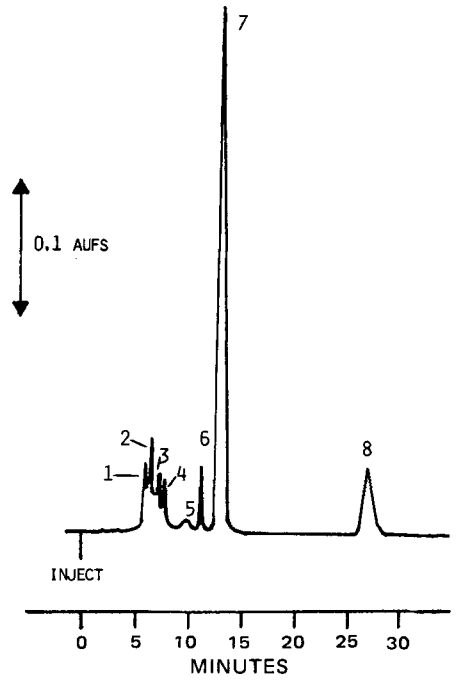


**Figure 2**—*Chromatograms of carbenicillin (1.944 mg/ml) spiked with 5–8% degradation products. The mobile phase was 35% methanol (pH 3.35) at 1.3 ml/min, and the detector was at 254 nm. Key: 1, carbenicillin penilloic acid; 2, carbenicillin penicilloic acid; 3, penicillin G penilloic acid epimer; 4, penicillin G penilloic acid epimer; 5, penicillin G penicilloic acid epimer; 6, penicillin G penicilloic acid epimer; 7, carbenicillin; and 8, penicillin G.*

## EXPERIMENTAL

**Apparatus**—The liquid chromatograph system consisted of a constant-flow pump[1], a fixed-volume loop injection valve[2], a 150 × 4.6-mm i.d. column packed in this laboratory by the method of Broquaire (7) with 5-$\mu$m Spherisorb ODS[3], a variable-wavelength UV detector[4], an electronic integrator[5], and a potentiometric recorder[6].

**Reagents**—Distilled water and distilled-in-glass methanol[7] were used. Production lots[8] of carbenicillin and penicillin G were used in the preparation of the degradation products by published methods (8). The remaining chemicals were reagent grade.

**Mobile Phase**—The mobile phases consisted of 35 or 37% methanol with the balance being 0.05 *M* $KH_2PO_4$ (v/v). Tetrabutylammonium bromide[9] (0.1%, w/v) was added as the ion-pair reagent. The pH was adjusted with 10% $H_3PO_4$, and the solution was degassed (laboratory vacuum) for 5 min prior to the initial use. Flow rates between 1.0 and 1.5 ml/min were employed.

**Carbenicillin Solutions**—A laboratory working standard of carbenicillin monosodium monohydrate dissolved in 0.02 *M* phosphate buffer (pH 7.0) was used for the liquid chromatographic work.

## RESULTS AND DISCUSSION

The HPLC resolution of the carbenicillin diastereomer pair and the subsequent quantitation problem were discussed previously (6). Diastereomers of other penicillins and cephalosporins were separated by HPLC (9), and the relationship of the mobile phase composition and pH on the degree of resolution was shown. The HPLC resolution of the carbenicillin diastereomers with a reversed-phase system and a micropar-

[1] Model II G, Laboratory Data Control, Riviera Beach, Fla.
[2] Model 210, Altex Scientific, Berkeley, Calif.
[3] Phase Separations Ltd., Hauppauge, N.Y.
[4] Spectromonitor III, Laboratory Data Control, Riviera Beach, Fla.
[5] Model E-1A, Shimadzu Scientific Instruments, Columbia, Md.
[6] Model A-25, Varian, Palo Alto, Calif.
[7] Burdick & Jackson Laboratories, Muskegon, Mich.
[8] Pfizer, Groton, Conn.
[9] Aldrich Chemical Co., Milwaukee, Wis.

ticulate column[10] first was observed in this laboratory in 1974[11], but the mobile phase pH effects were not understood fully at that time.

It was determined in the present study that the carbenicillin diastereomers can be either resolved or eluted as a single peak, depending on the mobile phase pH (Figs. 1A–1D). The conditions used to produce the chromatogram of Fig. 1B are more conducive to quantitation, either by manual or electronic methods. For example, Yamaoka *et al.* (6) were required to use a gravimetric method (cutting and weighing the HPLC recorder tracing) to obtain quantitative results.

Carbenicillin degrades *via* hydrolytic or decarboxylative routes. Penicillin G, which results from the carbenicillin side-chain decarboxylation, was present at a low level in the working standard of carbenicillin monosodium monohydrate, as evidenced by the peak near 17 min in Fig. 1B. Some carbenicillin samples were found to contain penicillin G sodium in amounts between 3.5 and 4.9% (10). The working standard also contained other process-related substances at low levels which eluted between 3 and 7 min.

To demonstrate assay noninterference, carbenicillin and penicillin G degradation products were synthesized by the methods reported previously (8), with one anomaly; the yield of penicillin G penicilloic acid as its monosodium salt monohydrate was significantly less than was reported, and this substance crystallized only with great difficulty. In addition, the conditions stated for the preparation of carbenicillin penicilloic acid apparently provide a mixture of epimers in an approximate 60:40 ratio, as evidenced by HPLC. Only one epimer crystallized.

The carbenicillin degradation products were essentially pure by HPLC[12]. The physical data obtained[13] were consistent with those reported (8, 11). A synthetically prepared mixture of carbenicillin, carbenicillin penicilloic acid, carbenicillin penilloic acid, penicillin G, penicillin G penicilloic acid, and penicillin G penilloic acid was subjected to chromatographic analysis. The chromatogram of Fig. 2 shows that carbenicillin was separated adequately from the degradation products used and that the carbenicillin peak could be quantitated easily.

A detector linearity study for carbenicillin concentrations, as its monosodium salt monohydrate, between 0.25 and 4.05 mg/ml in pH 7.0 phosphate buffer, showed an origin-oriented linear response at 254 nm. The calibration curve at 254 nm was characterized with a regression coefficient of 0.9998.

Reproducibility was demonstrated with 10 replicate analyses of the same solution (1.634 mg/ml) at 15-min intervals. The carbenicillin peaks were quantitated electronically by area, and the relative standard deviation was 0.79%.

Dissolving the carbenicillin working standard in the mobile phase adjusted to pH 7.0 caused a slow but steady degradation of carbenicillin. However, solutions of carbenicillin in 0.02 *M* phosphate buffer (pH 7.0) were stable for at least 5 hr.

This HPLC procedure is readily adaptable to the determination of penicillin G in carbenicillin samples and, with appropriate mobile phase modifications, has utility for the analysis of other penicillins.

[10] Micropak CH, 30 cm × 2.1 mm i.d., Varian, Palo Alto, Calif.

[11] Unpublished results.

[12] Penicillin G penicilloic and penilloic acids, and carbenicillin penicilloic acid to a lesser extent, tended to epimerize at the conditions (pH 3.35) used.

[13] Specific rotation, elemental analysis, IR spectra, equivalent weight, iodine titration, and Karl Fischer water determination.

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