compared with II that the effect on I would be insignificant. In addition, the analysis for intact II is sufficiently specific and quantitative for stability purposes. The known degradation product, *p*-aminophenol, and the impurity of I have different retention times compared with II (Table I). Good assay data have been obtained for several 5-year-old tablet samples. Since there was only an interest in the strength of the hydrocodone, no attempt was made to identify the decomposition products formed under stress.

For the content uniformity run, aliquots of the same 10 tablet samples were assayed by the HPLC and ion-pairing methods for II as paired comparison. Assay data for the ion-pairing analysis are generally lower compared with HPLC (Table V).

Through the utilization of the selected wavelength of maximum absorbance for II, this procedure is being routinely used as an automated simultaneous determination. The HPLC method reported in this study allows for rapid, specific, stability-indicating, and simultaneous quantitation of both substances in a two-component tablet formulation.

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High-Pressure Liquid Chromatographic Assays for Ticarcillin in Serum and Urine

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Abstract □ Rapid and sensitive high-pressure liquid chromatographic (HPLC) assays for ticarcillin in serum and urine have been developed. Sample pretreatment and optimized chromatographic conditions are presented for a C-18-bonded reversed-phase column used in an internal standard assay method. Ticarcillin has a retention time of ~5.3 min at a flow rate of 1.5 ml/min for a mobile phase of acetonitrile–aqueous 0.06 *M* sodium biphosphate, pH 2.05, (50.5:100). In a two-step extraction procedure, the ticarcillin extraction efficiencies from serum and urine were 76.1 ± 4.7 and 80.9 ± 3.2%, respectively. The assay sensitivity limit for ticarcillin in these fluids is ~1.0 μg/ml. A comparison is made of the HPLC and microbiological assay results for ticarcillin in 20 different but equally divided serum samples obtained from two volunteers.

Keyphrases □ Ticarcillin—high-pressure liquid chromatographic assays, serum and urine □ Urine—ticarcillin, high-pressure liquid chromatographic assays, serum □ Serum—ticarcillin, high-pressure liquid chromatographic assays, urine □ High-pressure liquid chromatography—ticarcillin in serum and urine

During the past decade, measurements of ticarcillin concentrations in clinical studies have been performed mainly by microbiological assays (1–9). A microtitration method (10, 11) has been applied to broth. Although numerous chemical and physical assay methods have been reported for the detection and assay of penicillins, they suffer from a variety of disadvantages. For instance, iodometric titration (12–15) cannot be applied to penicillins having unsaturated side chains. Spectrophotometric methods (16–18) are not very sensitive and a colorimetric assay (19–21) involving the reaction of hydroxylamine with penicillins in the presence of ferric ions to form a colored salt lacks both specificity and sensitivity, having a limit of ~20 μg/ml. A spectrofluorometric assay (22) has application only for fluorophoric penicillins. More recently, precise, convenient, and specific HPLC assays for various penicillins have been reported (23–29).

At the time the present study commenced there was, to the best of our knowledge, no HPLC assay reported for ticarcillin in biological fluids. This report presents a rapid, specific, and accurate HPLC assay for ticarcillin in human serum and urine.

EXPERIMENTAL

Materials—Powdered ticarcillin[1] (disodium salt) and carbenicillin[2], the internal standard, were used as received without further purification as standards. All solvents and reagents were analytical reagent grade, except for acetonitrile[3] which was HPLC grade, 190 nm cutoff.

Apparatus—The HPLC pump[4] was fitted with an injector[5] and variable wavelength detector[6] set at 210 nm and attenuated at 0.04 aufs. The signal was recorded either on a 10-mV recorder[7] at a speed of 0.5 cm/min or an integrator-plotter[8].

Columns—A prepacked 10- × 0.8-cm i.d. cartridge[9] containing a reversed-phase, C-18, chemically bonded to 10 μm of silica, and contained in a radial compression device[10] was employed at ambient temperature. A guard column[11] was placed between the injector and the column. Ti-

[1] Beecham Laboratories, Pointe Claire, Ontario, Quebec.
[2] Ayerst Laboratories Saint-Laurent, Quebec.
[3] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[4] Waters Associates Inc., Milford, Mass., model 6000A.
[5] Waters Associates, model U6K.
[6] Waters Association, model 450.
[7] Beckman 10 inch Recorder, model 1005.
[8] Waters Associates, Data Module, model 730.
[9] Waters Associates, Radial-Pak-A.
[10] Waters Associates, RCM-100 Radial Compression System.
[11] Whatman Inc., Column Survival Kit with Co-Pell ODS.

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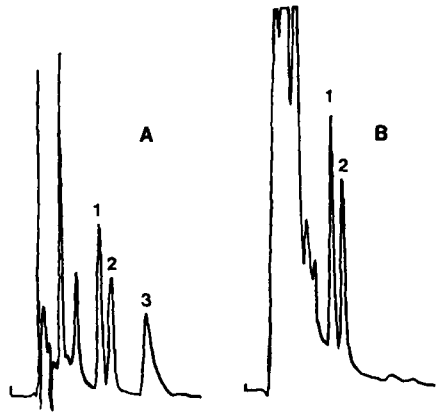

Figure 1—*Typical chromatograms of ticarcillin and carbenicillin (internal standard) in(A) serum and (B) urine sample. Peaks: (1) ticarcillin, (2) carbenicillin, and (3) endogenous material.*

carcillin sample and standard solutions containing the internal standard were injected in 10-, 25-, and 50-μl volumes with microliter syringes[12] into the injector valve.

Mobile Phase—A solution was prepared consisting of acetonitrile–aqueous 0.06 *M* sodium biphosphate (50.5:100), and the pH was adjusted to 2.05 with ~35 ml of 85% phosphoric acid. This solution was filtered and degassed by passage through a membrane filter[13] under reduced pressure. The mobile phase was pumped isocratically through the column at ambient temperature with a flow rate of 1.5 ml/min under a back pressure of ~350 psi/g.

Internal Standard—Aqueous carbenicillin sodium stock solution containing 59 mg/liter was prepared fresh weekly and stored at 4°. Accurate volume aliquots equivalent to 2.95 μg/50 μl of serum and 14.75 μg/250 μl of urine were used as internal standards in the assay for ticarcillin.

Standard Solutions—An aqueous stock solution of ticarcillin disodium (0.5113 g/100 ml) was prepared weekly and stored at 4°. Serial dilutions were performed daily to prepare six working standards in the range of 0–1400 μg/ml. Serum standards contained 200 μl of human control serum, 10 μl of a working ticarcillin standard solution, and 50 μl of the internal standard solution. Urine standards contained 500 μl of control human urine, 50 μl of a ticarcillin working standard, and 250 μl of the internal standard solution. In each instance calibration curves were initially constructed by plotting the integrated peak area ratios of the standard ticarcillin to internal standard against the corresponding ticarcillin standard concentration. Sample serums and urines were similarly prepared and the measured peak area ratios referred to the calibration curve to read the concentration of ticarcillin present.

Assay Method for Serum—A serum sample (200 μl) was transferred to a 12- × 75-mm polypropylene test tube, followed in succession by 50 μl of internal standard, and 50 μl of 1 *M* sulfuric acid. The tube contents were vigorously agitated with a mechanical mixer[14] for 5 sec, then 1.0 ml of ethyl acetate was added and the tube capped and shaken for 2 min prior to centrifugation[15] for another 2 min. The supernate was transferred to another similar tube and evaporated to dryness under reduced pressure at 35°. To this extract residue was added 200 μl each of methylene chloride and aqueous 0.04 *M* sodium biphosphate, pH 6.8. The tube contents were gently agitated for 1 min then centrifuged for 2 min. The top aqueous layer (~75%) was accurately transferred to another similar test tube and briefly shaken mechanically in a water bath at 35° to remove traces of methylene chloride. An accurate aliquot of 10–50 μl of this final sample solution was injected onto the RP-18 column.

Method for Urine—A 500-μl urine sample was treated in a manner similar to that described for serum, except for the volumes of solvents employed. A 250-μl volume of the internal standard solution was added to the 500-μl urine sample (or known dilution if required) and the mixture extracted once with 2 ml of ethyl acetate. The extract was evaporated to dryness as described for serum and the residue reconstituted in a mixture of 1.0 ml of methylene chloride and 1.0 ml of aqueous 0.04 *M* sodium biphosphate, pH 6.80. Beyond this point the sample was handled in the same manner as that for the serum.

[12] Hamilton Co., Reno, Nev., 25- and 100-μl syringes.
[13] Millipore Corp., No. FALP 04700, 1.0-μm pore.
[14] Vortex-Genie—Fisher Scientific Co.
[15] Sorvall, Newton, Conn. Model GLC-2, at 2000 rpm.

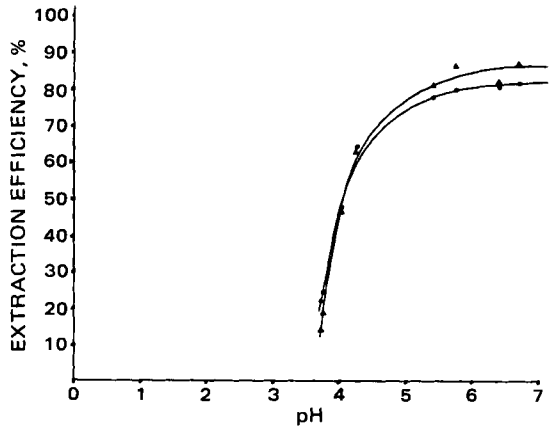

Figure 2—*Extraction efficiency of ticarcillin (●) and carbenicillin (▲) from urine as a function of the pH of the back-extraction phosphate buffer.*

Serum and Urine Collection and Assay—In an HPLC assay *versus* microbiological assay, nine 5-ml blood samples were obtained at specified intervals from each of two healthy volunteers following a 10-min intravenous infusion of a solution containing 2.0 g of ticarcillin disodium. Sera were separated by centrifugation and each was divided into two identical portions. One portion was subjected to a microbiological assay procedure analogous to that of Bannatyne and Cheung (30) and the other to the HPLC assay described. Triplicate determinations were made on each serum sample with both methods.

Concomitantly, urine samples were obtained from one of the volunteers receiving the 2.0-g dose of ticarcillin. Both time and urine volumes were recorded at each collection. Triplicate determinations were performed by the HPLC method only on each urine sample.

RESULTS AND DISCUSSION

The goals were to select a suitable reversed-phase column and mobile phase to separate the two penicillins, then develop a rapid and sensitive HPLC assay for ticarcillin in both serum and urine. The choices of columns and mobile phases studied were made on the basis of good peak symmetry, resolution, and short retention times suitable for measuring many biological samples in a relatively short period with good accuracy.

When the RP-8[16] column was used with several mobile phases, the ticarcillin was either poorly retained or eluted as asymmetric or fused peaks. Whether this result is due to varying degrees of ionization of the two carboxylic acid groups present, to the presence of sterisomeric structures, or to some other cause remains to be shown.

When these same solvent systems were used to elute ticarcillin from

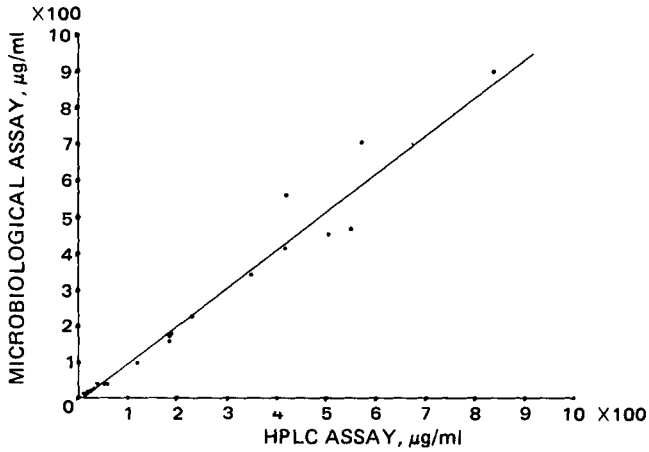

Figure 3—*Comparison of assays for ticarcillin in human urine. Identical samples were assayed by HPLC and microbiological methods. Linear regression analysis of this data yields the equation* $y = 1.0716x - 13.2182$, $r = 0.9809$.

[16] MPLC, Brownlee Labs, Santa Clara, CA 95050.

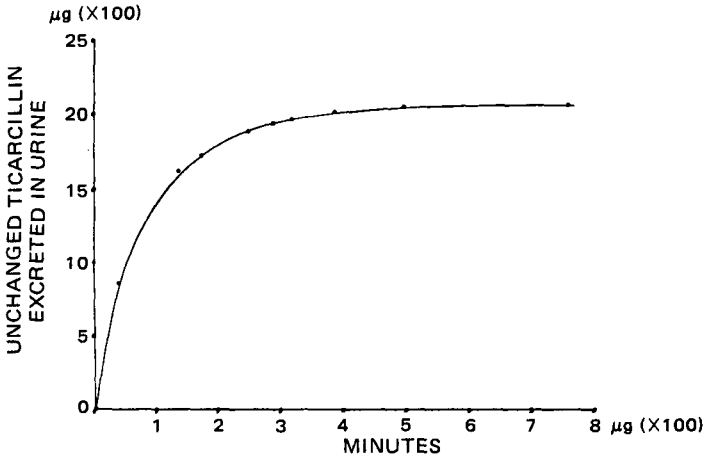

Figure 4—*Cumulative ticarcillin excreted in urine following slow intravenous infusion of a 2-g dose to a 70-kg normal male.*

a RP-2[16] column, retention times were improved, but the problem of fused or asymmetric peaks remains. Among the solvent systems tried was one comprised of 1 liter of aqueous 0.06 *M* sodium biphosphate and 125 ml of acetonitrile plus ~5 ml of 95% phosphoric acid to give a final pH value of 2.75. This mobile phase gave rise to a symmetrical peak for ticarcillin with a reasonable retention time of 5.50 min at a flow rate of 2.0 ml/min. Using the same conditions, except for the adjustment of pH values ranging from 5.0 to 2.0, the retention time was observed to increase from 1.5 to 11.0 min as the acidity increased. At pH values between 2.8 and 3.3, the ticarcillin peak was sharp but too close to the solvent peak resulting from each injection. At a pH of 2.75, both ticarcillin and carbenicillin gave symmetrical and well-resolved peaks having favorable retention times of 5.30 and 6.80 min, respectively.

Another reversed-phase column, RP-18[9], was found to work very well. Since ticarcillin is potentially a relatively polar compound having two carboxylic acid groups, its retention on the nonpolar RP-18 column could be increased through either ion-suppression or the ion-pairing techniques. The former was attempted due to its simplicity. Ticarcillin has a reported pKa value of between 2.70 and 2.73 (31); therefore, a new mobile phase consisting of 505 ml of acetonitrile and 1 liter of 0.06 *M* sodium biphosphate monobasic was adjusted by means of 85% phosphoric acid to a pH of 2.05. Use of this solvent system produced well-resolved, sharp symmetrical peaks at ~5.30 min and 6.00 min for ticarcillin and carbenicillin, respectively, at a flow rate of 1.50 ml/min (Fig. 1). If the pH was 2.20, ticarcillin eluted as two fused peaks at 4.42 and 4.85 min. These observations underscore the necessity of having the mobile phase accurately buffered to a pH of 2.05.

Serum and Urine Assays—Assays were developed using simulated samples. Penicillins lack a strong chromophoric group in their structure. The wavelength of maximum absorption for ticarcillin occurs slightly below 200 nm, and this region would give the highest sensitivity for measurement. However, several endogenous substances in these biological fluids also give rise to absorption leading to interference in this region. Therefore, in order to obtain specificity and maximum sensitivity for ticarcillin, these interferences had to be eliminated. Several approaches (32) were taken to solve this problem. First, several deproteinizing agents such as acetonitrile, cold methanol, and 5% trichloroacetic acid in methanol were investigated. Second, drug extractions using various organic solvents alone were attempted. Finally, organic solvent extraction followed with back-extraction into aqueous buffers was attempted. The protein precipitation method met with little success. The one-step organic solvent extraction of ticarcillin and carbenicillin from serum and urine at pH 2.0 using four volumes of ethyl acetate showed some promise but also extracted interfering endogenous materials. These were slow to elute from the column, interfered with the separation of subsequent sample, and necessitated frequent column cleaning.

A back-extraction of the ethyl acetate extract with 0.04 *M* sodium biphosphate, pH 6.8, to separate the ticarcillin and the internal standard from these biological interferences proved very successful. However, when the UV detector was set at 210 nm, an intense peak appeared (t_r = 5.52 min) which severely interfered with the quantitation of the ticarcillin peak. It was subsequently identified as a peak due to ethyl acetate in the aqueous buffer extract. This problem was circumvented by evaporating the ethyl acetate extract to dryness under reduced pressure at 35°, then adding to the residue equal 0.2-ml volumes of methylene chloride and aqueous 0.04 *M* sodium biphosphate (pH 6.8), and vigorously agitating for 1 min prior to centrifugation for 2 min to separate the immiscible phases. The interfering ethyl acetate remained in the methylene chloride phase, while the two penicillins passed into the aqueous buffer layer. A small trace of interference by methylene chloride was readily removed from the aqueous layer by a brief vigorous agitation at 35° prior to the injection of an aliquot of this phase.

Extraction Efficiency—This was expressed as a percent recovery, defined here as the quotient of the peak height of a ticarcillin standard extracted from the biological fluid divided by the peak height of the corresponding amount of drug prepared as a standard solution in the mobile phase and injected directly, all multiplied by 100. The pH value of the aqueous 0.04 *M* sodium biphosphate used in the back-extraction step had a very important effect on the extraction efficiency of both ticarcillin and carbenicillin as shown in Fig. 2. The recovery of both drugs was optimized at >pH 6.0, and the ratio of the ticarcillin peak height divided by that for carbenicillin became constant. A pH >7 was avoided due to possible base-catalyzed degradation of these penicillins. The mean recovery of six replicate serum standards of ticarcillin and carbenicillin was 76.1 ± 4.7% (*CV* = 6.2%) and 76.3 ± 4.5% (*CV* = 5.8%), respectively. The mean recovery of nine urine standards was 80.9 ± 3.2% (*CV* = 4.0%) and 84.5 ± 5.3% (*CV* = 6.3%), for ticarcillin and carbenicillin, respectively.

Accuracy of the HPLC Assays—The quantitation was based on the internal standard method, *i.e.*, the linearity of the plot of the peak area ratios of ticarcillin standards divided by carbenicillin, *versus* the known concentrations of ticarcillin used in the preparation of the standards in mixed human control serum and urine. The linearity of this plot was determined by linear regression analysis using six serum and six urine ticarcillin standards with each standard concentration measured in duplicate. The results were found to be: $y = 0.0061 + 0.0371x$ ($r = 0.9998$), and $y = 0.0150 + 0.343x$ ($r = 0.9995$), for serum and urine standard curves, respectively. The concentration of ticarcillin in the biological sample was obtained by calculating the peak area ratio, substituting the value for y in the appropriate equation, and solving for x.

HPLC and Microbiological Assay Comparison—In the comparison of the HPLC and microbiological assays for ticarcillin in identical *in vivo* serum samples, it is evident from Fig. 3 that these methods are in good agreement having an excellent correlation coefficient ($r = 0.9809$). The overall mean of the percent difference between the microbiological and HPLC values was 13.1%, when all serum samples were considered. One reason for this seemingly large difference is due to the fact that a broad range of ticarcillin concentrations (0–1000 μg/ml) in serum was compared. It must be remembered that the microbiological assay was less accurate at the lower end of the concentration range, *i.e.*, at <50 μg/ml, while the HPLC method was more accurate at <200 μg/ml.

Precision—One simulated ticarcillin serum sample and one urine sample were treated and extracted in the manner outlined earlier. Six replicate injections of each sample were made onto the RP-18 column, and the integrated peak area ratios were calculated. The mean peak area ratios of ticarcillin–carbenicillin were 0.711 ± 0.010 (*CV* = 1.3%) and 1.148 ± 0.013 (*CV* = 1.2%) for serum and urine samples, respectively.

The minimum quantitation limit was ~1 μg/ml for ticarcillin in these biological fluids based on a signal to noise level ratio of three.

Ticarcillin urine levels from one human volunteer were measured with the HPLC assay described. The cumulative amount of unchanged ticarcillin excreted is shown in Fig. 4. Over a period of 12 hr, 103.5% of the administered intravenous dose was measured in the urine. This value agrees well with the 6-hr urine excretion of 99% of a dose administered intramuscularly (33). No degradation product of ticarcillin was detected in any urine sample assayed.

Preliminary pharmacokinetic results (32) obtained using the developed HPLC assay are in very good agreement with those reported in the literature using other assays (34, 35). Further pharmacokinetic studies employing this assay are in progress.

The results indicate that a rapid, convenient, sensitive, specific, and accurate HPLC assay for ticarcillin in serum and urine has been developed and shown to have potential application for pharmacokinetic and clinical studies with this penicillin.

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Concomitant Adsorption and Stability of Some Anthracycline Antibiotics

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Abstract □ Using liquid scintillation counting and liquid chromatographic techniques, it has been demonstrated that the anthracycline antibiotics, doxorubicin hydrochloride, *N*-trifluoroacetyladriamycin-14-valerate, and *N*-trifluoroacetyladriamycin-14-octanoate, can be strongly adsorbed to the walls of containers, depending on the nature of both the container material and the solute. It also has been shown that the esters are unstable in the chemical growth media commonly used in cell culture studies. Both the adsorption and stability effects are suggested as being factors which should be carefully considered in interpretation of the *in vitro* and perhaps *in vivo* activities of the anthracycline esters.

Keyphrases □ Anthracyclines—concomitant adsorption and stability, esters, cell culture studies □ Antibiotics—anthracyclines, concomitant adsorption and stability, cell culture studies □ Adsorption—concomitant adsorption and stability of some anthracyclines, cell culture studies

The anthracycline antibiotic, doxorubicin hydrochloride (I), has a wide range of antitumor activity (1). Although I given singly or in combination is now used extensively in the treatment of a variety of tumors (2, 3), its clinical value is limited due to its acute myelotoxicity and to the frequent development of irreversible cardiomyopathy [normally observed when the accumulated dose is >550 mg m^{-2} (4)]. The use of structural analogs of I has been proposed (5, 6) as a means of obviating these two effects; in this respect particular interest has been given to the proposed use of the esters of I and its *O*- and *N*-acetylated derivatives (7–14).

During a study examining the uptake of I and its analogs into tumor cells *in vitro* using both cell monolayers and suspensions, effects were observed on the mass balances of some radiolabeled anthracyclines which could only be reconciled in terms of solute adsorption to container walls. Accordingly, the role of adsorption and stability on the solution properties of I and two of its *N*-trifluoroacetyl esters (*i.e.*, the 14-valerate and the 14-octanoate) have been investigated more completely. The present report presents the results found in this investigation.

EXPERIMENTAL

Chemicals—Anthracycline antibiotics were obtained commercially[1]. ^{14}C-Labeled antibiotics were synthesized as described previously (15). Samples of solid I, *N*-trifluoroacetyladriamycin-14-valerate (II), and *N*-trifluoroacetyladriamycin-14-octanoate (III) had specific activities of 17.1, 22.5, and 28.3 μCi $\mu mole^{-1}$, respectively.

[1] Adria, Ohio.

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