a doubling time of 10–12 and 18–24 hr, respectively. The solutions were diluted to a stock solution of 10^{-3} *M* with phosphate-buffered saline, sterilely filtered, and aseptically diluted by half-log increments. Each concentration (0.7 ml) was added to duplicate 13- × 75-mm test tubes. Cells from logarithmically growing stock culture were suspended in prewarmed medium[4] supplemented with 10% dialyzed fetal calf serum, 10 m*M* (morpholine-propanesulfonic acid, and 20 m*M* [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid]. Cell suspension (1.8 ml) was added to each tube. The tubes were incubated upright at 37° in a warm room or dry incubator. Under these conditions, L-1210 cells grew exponentially 15- to 25-fold from an initial density of 2–2.5 × 10^4/cm^3; CCRF-CEM cells grew exponentially 8- to 10-fold. After 48 hr (for L-1210 cells), or 72 hr (for CCRF-CEM cells), the incubation was terminated and the cell densities were determined[5]. The degree of proliferation of each 2-ml culture was expressed as the ratio of the final cell density to the initial cell density; this index was plotted against the drug concentration employed. The concentration of drug which depresses the ratio to 50% of control (the IC_{50}) was graphically determined. For the clinically effective drug, 5-fluorouracil, IC_{50} = 1.9 × 10^{-6} *M* for CCRF-CEM cells in culture.

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[5] Determined by using a Coulter Counter.

High-Pressure Liquid Chromatographic Assay of Cloxacillin in Serum and Urine

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Abstract □ Two rapid, specific, and sensitive high-pressure liquid chromatographic (HPLC) assays were developed for cloxacillin in serum and urine. A reversed-phase column (RP-8) was selected for use with two different sets of HPLC conditions and sample pretreatment procedures. Cloxacillin extraction efficiencies are reported from serum and urine. Equations are presented for linear relationships between peak height or peak area ratios of cloxacillin to nafcillin (internal standard) and the cloxacillin concentration over a range of 0–80 μg/ml. The sensitivity limit of these assays was ~0.3 μg/ml of a standard solution for one method and 0.05 μg/ml for the other HPLC assay.

Keyphrases □ Cloxacillin—high-pressure liquid chromatographic assay, serum, urine, nafcillin □ Nafcillin—cloxacillin, high-pressure liquid chromatographic assay, serum, urine □ High-pressure liquid chromatography—cloxacillin in serum and urine □ Penicillins—high-pressure liquid chromatography of cloxacillin

Cloxacillin sodium (I), [3-(*o*-chlorophenyl)-5-methyl-4-isoxazoyl] penicillin sodium, is a semisynthetic penicillin synthesized in 1962 (1). It can be administered both parenterally and orally. Like other penicillins, I is sensitive to nucleophilic and electrophilic attack catalysed by general bases and acids, respectively. Maximum stability of

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I occurs at pH 6.3 (2). The pKa of the carboxylic hydrogen is reported as 2.70–2.73 in water at 25° (3). Cloxacillin is 94–96% bound to serum proteins (4).

Previously reported assays for I include those based on spectrophotometry (5–10), titrimetric methods (6), microbiological methods (6, 7), an electrophoretic method (11), a polarimetric procedure (12), and an HPLC method for aqueous solutions (13).

Paper chromatograms of urine and serum samples following oral administration of I showed a similar ratio of unchanged I and a metabolite (14). Intramuscular injection of I is reported to give rise to metabolite formation as well. Cole *et al.* (15) reported that 21.6% of I appeared as the penicilloic acid metabolite in urine collected within 12 hr of a 500-mg oral dose but that none was found at half this dose.

Prompted by the apparent absence of any reported HPLC assay for cloxacillin in human serum or urine, two such methods were developed and are reported in this paper. These methods were developed for clinical studies and preliminary pharmacokinetic evaluation of I (16).

EXPERIMENTAL

The two distinct sets of HPLC assay conditions reported here will be referred to as HPLC Systems I and II. System I: RP-8 column; mobile phase, methanol–aqueous 0.04 *M* NaH_2PO_4 (4.1:5.9), pH 4.5; flow rate, 1.1 ml/min; detection wavelength, 225 nm; chart speed, 0.508 cm/min; retention time, cloxacillin, 8.75 min and nafcillin, 15.80 min. System II: RP-8 column; mobile phase, acetonitrile–aqueous 0.04 *M* NaH_2PO_4 (6.2:20), pH 4.5; flow rate, 1.6 ml/min; detection wavelength, 210 nm; chart speed, 0.508 cm/min; retention time, cloxacillin, 5.10 min and nafcillin, 6.60 min. Likewise, two different sample pretreatment procedures were used and are referred to as Procedures A and B.

Materials—A 10-cm × 4.6-mm i.d. stainless steel column[1] containing a chemical bonded reversed-phase (C_8) on 10-μM silica was selected. The modular HPLC system was comprised of a pump[2], an injection valve[3], a guard column[4], a variable wavelength UV-detector[5], and an integrator-plotter[6]. Other equipment used included a centrifuge[7] and mechanical mixer[8]. All inorganic buffer salts and acids were analytical grade and were dissolved in reagent grade water[9]. Organic solvents were acetonitrile[10] and methanol[10].

Mobile Phases I and II were deaerated and filtered through 1.0-μM pore membranes[11] under reduced pressure.

Standard Solutions—Cloxacillin sodium[12] (I) and nafcillin sodium[13] (II) were used as received for weekly preparation of stock standard and stock internal standard solutions, respectively. Human control serum[14] and control urine were used in the daily preparation of working standard solutions as well as simulated samples. Standard solutions of I were prepared in water, control serum, and control urine as follows: to each of seven appropriately labeled tubes[15] was added in succession 0.5 ml of water (or urine or serum), 0.01 ml of one of the working standards of I in the 0–54.3-μg range, and 0.05 ml (equivalent to 12.0 μg of II) of internal standard solution. The contents of each tube were thoroughly mixed by the mechanical mixer. These standards and similar simulated samples were subjected to one or the other of the pretreatment Procedures A or B, to compensate for recovery losses and other systematic errors.

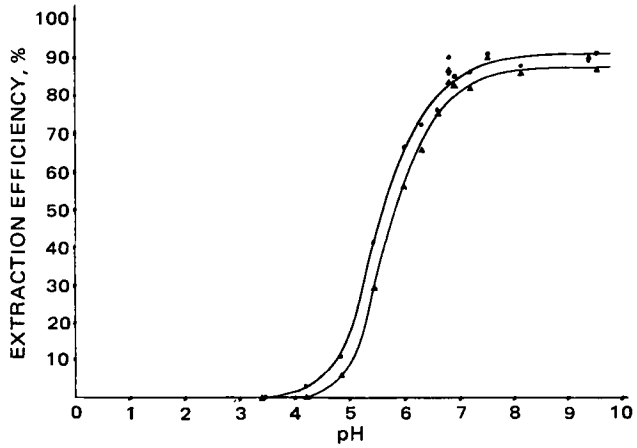

Figure 1—*Extraction efficiency of cloxacillin (●) and nafcillin (▲) from urine as a function of pH of the 0.04-*M *sodium dihydrogen phosphate buffer.*

Standard and Sample Pretreatment Procedures—*Procedure A*—Protein precipitation was performed by adding dropwise 0.5 ml of acetonitrile to a 0.5-ml sample of serum (or urine) in a small tube. This was followed by agitation for 20 sec by the mixer and centrifugation at 3000 rpm for 1 min. The clear supernate was filtered[16], and 10 μl of the filtrate was injected onto the column.

Procedure B—A drug extraction and back extraction procedure was performed as follows: to 0.5 ml of serum (or urine), in a small test tube, was added 0.05 ml of internal standard solution, equivalent to 12.0 μg of II. Then 0.05 ml of 1 *M* H_2SO_4 was added and the tube was mixed for 5 sec. Two milliliters of methylene chloride[17] was added and the tube was shaken for 2 min prior to centrifugation at 2000 rpm for 2 min. The organic layer was transferred to a second tube containing 1.0 ml of aqueous 0.04 *M* NaH_2PO_4, pH 6.80. This tube was stoppered and the shaking and centrifugation steps were repeated. An aliquot equivalent to ~75% of the aqueous layer containing the penicillins was transferred to a third tube, which was agitated vigorously on the mixer for 20 sec to dispel the volatile methylene chloride carried over in the aqueous layer. Injections (10 μl) of the final assay solution were used. Either integrated peak area ratios or peak height ratios of I–II were calculated and referred to a standard curve similarly constructed with standard solutions of I in accordance with the internal standard assay method. The sample concentrations were reported as micrograms of I per milliliter of serum or urine.

Precision and sensitivity limits were estimated for these assays.

RESULTS AND DISCUSSION

Preliminary trials were made using three reversed-phase columns[18] and mobile phases consisting of various ratios of aqueous buffers and either methanol or acetonitrile. Simple mixtures of water and methanol or acetonitrile proved inadequate for these assays. The effect of buffer pH was studied (16). The best results were obtained with an RP-8 column using aqueous 0.04 *M* NaH_2PO_4, pH 4.5, blended with methanol or acetonitrile in HPLC Systems I and II, respectively. These systems provided adequate resolution at desirable retention times. The wavelength of detection was selected to give maximum sensitivity consistent with minimum interference from trace endogenous biological materials. The method of sample pretreatment was developed to minimize interferences arising from the biological fluids.

Pretreatment Procedure A involving a simple protein precipitation step produced good results when HPLC System I was used. However, the addition of acetonitrile diluted the sample and, hence, decreased the level of sensitivity for I in the method. Procedure B involved the extraction of the undissociated acid forms of penicillins I and II with methylene chloride, followed by back-extraction of the ionized forms into aqueous phosphate buffer, pH 6.8. This procedure reduced the level of endogenous

[1] MPLC Concept RP-8 column and holder, Brownlee Labs, Santa Clara, CA 95050.
[2] Model 6000A, Waters Associates Inc., Milford, MA 01757.
[3] Model U6K, Waters Associates, Milford, MA 01757.
[4] Column survival kit packed with CO: Pell ODS (No. 6561-403), Whatman, Inc., Clifton, NJ 07014.
[5] Model 450, Waters Associates, Milford, MA 01757.
[6] Data module, model 730, Waters Associates, Milford, MA 01757.
[7] Sorvall Centrifuge, model GLC-2 with head adapter HL-4 and No. 575 tube holders, Du Pont Co., Wilmington, DE 19898.
[8] Vortex-Genie, Cat. No. 12-812-VI, Fisher Scientific Co.
[9] Millipore Corp., Bedford, MA 01730.
[10] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[11] Gelman Instrument Co., Ann Arbor, MI 48106.
[12] Beecham Laboratories, Pointe Claire, Quebec, Canada.
[13] Wyeth Limited.
[14] Grand Island Biological Co., Grand Island, NY 14072.
[15] 12-mm × 75-mm polypropylene or glass tubes closed with polyethylene caps.
[16] Type FA, Teflon, 0.5-μm pore diameter, Millipore Corp.
[17] Methylene chloride, HPLC-grade, UV-Cutoff 230 nm, Caledon Laboratories, Ltd.
[18] Brownlee Labs, Santa Clara, CA 95050, MPLC Concept RP-2, RP-8, and RP-18, 10-cm × 4.6-mm i.d. columns.

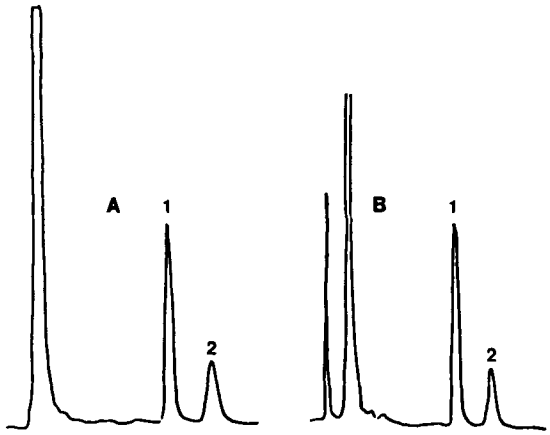

Figure 2—*(A) Representative chromatogram of a serum extract of (1) cloxacillin (9.04 min) and (2) nafcillin (11.42 min) on a 25-cm × 4.6-mm i.d. RP-8 column with a column guard. (B) Same as A except the column guard was removed: (1) cloxacillin (7.22 min), and (2) nafcillin (9.4 min).*

material interference and enhanced the sensitivity of the assay for I and II through the use of a detection wavelength of 210 nm.

Figure 1 shows the pronounced effect of buffer pH on the extraction efficiency of penicillins I and II in pretreatment Procedure B. A pH of 6.80 gave near optimal recovery and remains within the pH range of compatibility for these reversed-phase columns. A greater level of interference by endogenous biological materials occurs as the buffer pH becomes >pH 7.0 (16). The methylene chloride volume used in Procedure B also markedly affects the overall extraction efficiency of I and II. A 1:1 volume ratio of methylene chloride–serum produced a troublesome gelatinous globule due to proteins present. Although volume ratios of 4:1 or 5:1 gave similar and good drug recoveries, 6:1 or larger ratios caused lower recoveries of these drugs (16). The 4:1 volume ratio of methylene chloride–serum was selected for the sample (or standard) serum pretreatment Procedure B. The length of exposure of drugs I and II to sulfuric acid (Procedure B) is critical. This exposure to acid must be minimized and fixed at a value of <5 min. It has been found that ~1.5% of cloxacillin and 6.4% of nafcillin are degraded during 5 min when exposed to this strong mineral acid. Fortunately, this exposure to acid can easily be reduced if only a few serum samples are extracted at one time. The absence of degraded I and II peaks on HPLC chromatograms of samples pretreated by Procedure B indicates that insignificant, if any, degradation occurred during this short extraction period.

Figure 2 shows a representative chromatogram of cloxacillin and internal standard, nafcillin, extracted by Procedure B and chromatographed by System II, except that a longer column[19] of the same type packing was used. Figure 2 also shows the influence of the guard column. Figure 3 shows a typical chromatogram obtained with the shorter RP-8 column[18], using otherwise identical conditions. Note the small peak at 3.70 min due to residual methylene chloride, which was not adequately removed from this sample as it was in the normal procedure for Procedure B. This shorter RP-8 column had an advantage in that time and solvents were saved due to the shorter retention times for penicillins I and II.

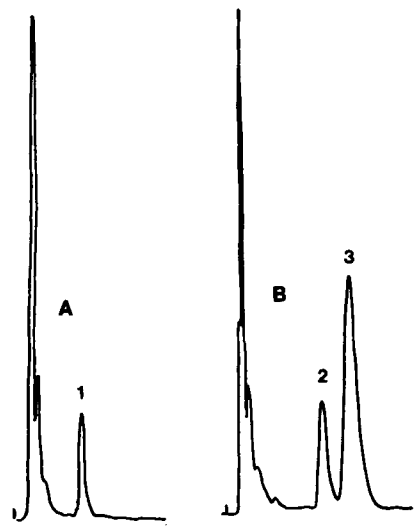

Figure 3—*Cloxacillin extracted from serum and chromatographed on a 10-cm × 4.6-mm i.d. RP-8 column. (A) Blank serum extract showing Peak 1 due to unremoved methylene chloride (3.70 min); (B) serum extracted cloxacillin (Peak 2, 5.3 min) and nafcillin (Peak 3, 6.85 min).*

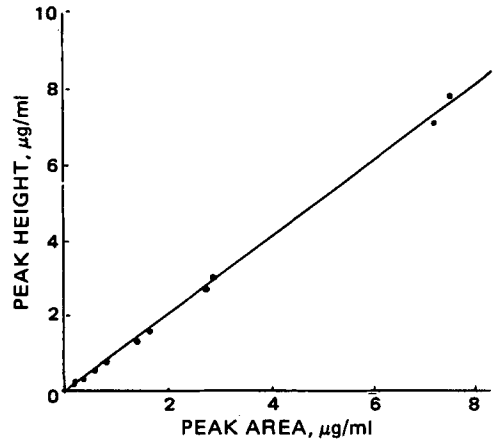

Figure 4—*Comparison of the serum cloxacillin concentrations from two human males as assayed by the internal standard method using peak height ratio and peak area ratio of cloxacillin to nafcillin. Linear regression analysis of this plot yields the equation* $y = 1.0117x - 0.0452$, *with a coefficient of correlation,* $r = 0.9990$. *A 250-mg iv dose of cloxacillin was given to each volunteer.*

The mean absolute extraction efficiencies for six replicate cloxacillin standards using Procedure B were 83.4 ± 1.4% *SD*, coefficient of variation (*CV*) = 1.6 and 91.4 ± 3.5%, *CV* = 3.8%, from serum and urine standards, respectively (16). Similarly determined mean extraction efficiencies for nafcillin (internal standard) were 78.9 ± 1.4%, *CV* = 1.8% and 84.5 ± 2.8%, *CV* = 3.3% for serum and urine standards, respectively. Neither serum nor urine standards containing both penicillins I and II gave rise to any extra peaks which could not be accounted for when the assay involved the pretreatment Procedure B with HPLC System II. This suggests the absence of any significant *in vitro* acid degradation of I and II. These reproducible high recoveries from both biological fluids ensure rather acceptable sensitivity for these assays of cloxacillin.

The precision of the HPLC System II was determined using a simulated serum sample containing 5 μg of I and 1.5 μg of II as well as 0.5 ml of control human serum. Sample pretreatment Procedure B was used and six replicate 10-μl injections of the final assay solution were assayed according to HPLC System II in which peak height ratios were calculated. The mean peak height ratio of I–II was 3.273 ± 0.032, *CV* = 1.0%.

The minimum quantitative limit, defined here as a signal–noise ratio of 3, for cloxacillin in an aqueous standard was ~0.3 μg/ml using HPLC System I and 0.05 μg/ml using HPLC System II. These do not represent overall assay sensitivity limits, since no pretreatment procedures were employed.

In the assay of cloxacillin using sample pretreatment Procedure B and HPLC System II, either peak height ratios or integrated peak area ratios of I–II can be used. Twelve serum samples were obtained from two male humans after different times following infusion of 250 mg iv of cloxacillin. Duplicate aliquots of each sample were extracted using Procedure B and assayed with HPLC System II. Figure 4 shows a comparison of the two methods of plotting, through regression analysis[20], in which the coefficient of correlation ($r = 0.9990$) indicates excellent agreement. Therefore, either I–II ratio can be used in the preparation of the internal standard method calibration curve for I. Figure 4 also confirms the excellent reproducibility of this assay over the concentration range used. The equation obtained by regressional analysis of the serum calibration curve, using peak height ratios of I–II *versus* concentration of I in six cloxacillin serum standards assayed by the same procedure, was $y = 0.591x + 0.0162$, ($r = 0.9976$).

Since both penicillins were somewhat more efficiently extracted

[19] Lichrosorb RP-8 packing in a HiBar II column, 25-cm × 4.6-mm i.d., EM Lab Inc., E. Merck, Darmstadt, W. Germany.

[20] Hewlett-Packard calculator model 9100A; Plotter, model 9125B; and program cards, model 9100.

(Procedure B) from urine than from serum, the calibration curves for I in these two biological fluids differed slightly. These calibration curves can be represented by equations obtained through linear regressional analysis: $y = 0.0437x + 0.0070$, ($r = 0.9991$) and $y = 0.0462x + 0.0270$, ($r = 0.9985$) for serum and urine cloxacillin standards, respectively. In these equations, y equals the integrated peak area ratio of I–II, and x equals the cloxacillin standard concentration in the serum or urine. HPLC System II was used to obtain these calibrations. The cloxacillin concentration covered the 0–80 μg/ml range of biological fluid.

The specificity of either HPLC System I or II is indicated by the fact that no endogenous material, metabolite of I, or degradation product of I or II gave peaks having the same retention times of I or II in chromatograms of *in vivo* serum or urine or of simulated *in vitro* serum or urine samples. The same was true for aqueous solutions of I or II deliberately degraded under acidic or alkaline conditions (16). No interfering peaks were detected in HPLC System II from extracts of human or rabbit serum or of human urine following intravenous administration of cloxacillin. Many other drugs which might be administered during cloxacillin therapy failed to interfere with this HPLC assay. The specific drugs tested for interference were penicillin G, amoxicillin, ticarcillin, carbenicillin, cephalexin, ampicillin, theophylline, acetaminophen, hydrocortisone acetate, vitamins A, C, D and E, and chloramphenicol (16). While this is not an exhaustive list, it represents many drugs that might be found in conjunction with I in urine or serum. Diazepam might partially interfere with this HPLC assay if its serum level is high (16).

The assay method involving pretreatment Procedure A and HPLC System I was initially developed to assay cloxacillin in human serum and urine but has also been used to determine I in rabbit plasma and serum. The same assay was used to establish that 51.35% of an intravenous dose of cloxacillin was excreted unchanged in the urine of one 75-kg human male volunteer within 24 hr. An extra peak was observed not previously seen in *in vitro* urine samples of cloxacillin. The peak may represent a metabolite, since it is not identical to any acidic or alkaline-degraded I peak. Cloxacillin had half-life values ($t_{1/2}$) of 26.9 and 26.5 min in two healthy human male volunteers who received intravenous doses of I (16). These values are in reasonable agreement with a reported value of 33 min in normal human adults (17). The overall elimination rate constants determined in the same two persons were 0.0258 min^{-1} and 0.0262 min^{-1} (16) and compare favorably with a reported value of 0.021 ± 0.003 min^{-1} (17).

In summary, two successful HPLC assays, together with serum and urine sample pretreatment procedures, have been developed. Pretreatment Procedure A used with HPLC System I, while slower, is superior for the resolution of peaks for I and II from each other and all biological endogenous materials or degradation compounds of I or II. The assay including pretreatment Procedure B and HPLC System II is preferred for the rapid assay of unchanged cloxacillin in urine and serum. This assay is specific, sensitive, rapid, precise, and has sufficient accuracy for the intended purposes.

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