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DETERMINATION OF AMDINOCILLIN IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TEH LO LEE* and MARVIN A. BROOKS

Department of Pharmacokinetics and Biopharmaceutics, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

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SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of amdinocillin (formerly mecillinam) in human plasma and urine. The assay is performed by direct injection of a plasma protein-free supernatant or a dilution of urine. A 10-μm μBondapak phenyl column with an eluting solvent of water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.5) was used, with UV detection of the effluent at 220 nm. Azidocillin potassium salt [potassium-6-(D-(−)-α-azidophenyacetamido)-penicillanate] was used as the internal standard and quantitation was based on peak height ratio of amdinocillin to that of the internal standard. The assay has a recovery of 74.4 ± 6.3% (S.D.) in the concentration ranges of 0.1—20 μg per 0.2 ml of plasma with a limit of detection equivalent to 0.5 μg/ml plasma. The urine assay was validated over a concentration range of 0.025—5 mg/ml of urine, and has a limit of detection of 0.025 mg/ml (25 μg/ml) using a 0.1-ml urine specimen per assay.

The assay was applied to the determination of plasma and urine concentrations of amdinocillin following intravenous administration of a 10 mg/kg dose of amdinocillin to two human subjects. The HPLC and microbiological assays were shown to correlate well for these samples.

INTRODUCTION

Amdinocillin [I], 6-β-{[(hexahydro-1H-azepin-1-yl)-methylene]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (Fig. 1) developed by Leo Pharmaceuticals Products, Hälsingborg, Denmark is one of a new class of semisynthetic β-amidino penicillins [1]. The structure of the compound differs from the classical penicillins by possessing an amidino group at the C-6 position of the penicillanic acid moiety instead of an acylated amino group. It has high activity against gram negative organisms, such as *Escherichia coli* [2].

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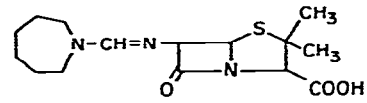
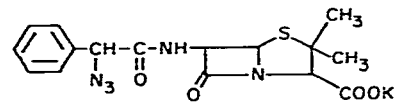
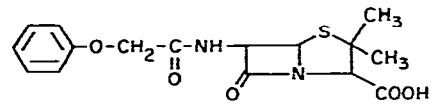
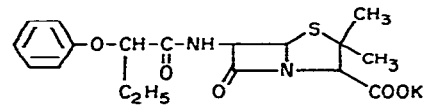
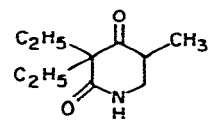
Compound	Name	Structure
I	Amdinocillin, 6-β-{[(hexahydro-1H-azepin-1-yl)-methylene]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]heptane-2-carboxylic acid	
II	Azidocillin potassium salt, potassium-6[D-(−)-α-azidophenyl-acetamido]-penicillanate	
III	Penicillin V, 6-phenoxyacetamido-penicillanic acid	
IV	Propicillin, α-phenoxypropyl-penicillin	
V	Methyprylon, 2,4-dioxo-3,3-diethyl-5-methylpiperidine	

Fig. 1. Chemical names and structures of compound I to V.

Routine determination of [I] for pharmacokinetic studies [3] in plasma and urine are often performed by microbiological assay [4]. A thin-layer cylinder-plate method for differentiation of amdinocillin pivoxil and amdinocillin has also been reported [5].

Spectrophotometric methods based on the formation of strongly UV absorbing (325—345 nm) imidazol-5-(4H)-one derivatives of amdinocillin by reaction of [I] with imidazole [6] or glycine [7] and mercuric chloride have been reported for use in aqueous solutions [6] and for studies involving the degradation of [I] [7]. The glycine reaction which required the β-lactam ring and the amidino side chain is highly specific for [I] in the presence of degradation products and other penicillins.

High-performance liquid chromatographic (HPLC) assays to measure [I] as the intact compound for stability determination [8] and as the imidazol-5-(4H)-one derivative formed with imidazole and mercuric chloride in biological fluids have been reported [9]. The HPLC assay in biological fluids utilizes post-column derivatization with air segmentation and is specific for penicillins with

an intact β-lactam ring, however, penicilloic acids of penicillins (i.e., ampicillin and amoxycillin) with free amino groups also react to some extent [9]. HPLC procedures have also been reported for the analysis of intact [I] in urine [10, 11] and plasma [11], and the pharmacokinetics of [I] in human study [12].

The present HPLC assay for plasma and urine utilizes reversed-phase chromatography on a μphenyl column with UV detection at 220 nm and is similar to that previously described [11]. Sample preparation for plasma has been simplified by elimination of the evaporation step. In addition, clean extracts are obtained by the inclusion of a diethyl ether wash of the acetonitrile protein-free filtrate prior to HPLC analysis. The urine assay also performed on the μphenyl column utilizes direct analysis of a diluted aliquot of the sample similar to that previously described [10,11]. The internal standard used in the plasma and urine assays is azidocillin potassium salt [II] (Fig. 1). The assay was applied to the determination of plasma concentrations and the urine excretion of [I] in two human subjects following intravenous administration of 10 mg of [I] per kg. The HPLC data obtained in these studies correlated fairly well with those of the bioassay [13], demonstrating that the two procedures are equivalent.

EXPERIMENTAL

Column

The column used was a prepacked 30 cm × 4 mm I.D. stainless-steel column containing 10-μm μBondapak phenyl packing (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a guard column with Bondapak phenyl/corasil as packing material (Waters Assoc.). An LDC Model Spectromonitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) variable-wavelength UV detector was used for quantitation at 220 nm. A back-pressure coil (approximately 3 m × 1.5 mm O.D. × 0.3 mm I.D. Altex PTFE tubing) was connected to the sample outlet of the flow-cell. The mobile phase used for isocratic reversed-phase chromatography in both the plasma and urine assays was a mixture of water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.5). Changes in retention time or resolution of [I] and [II] caused by the endogenous substances contained in the sample extracts may require small changes (approximately 5%) in the ratio of methanol and water in the mobile phase. The chromatographic system was operated at ambient temperature, with a flow-rate of 2.0 ml/min at a head pressure of $2.3 \cdot 10^3$ p.s.i. (15.9 MPa). Under the above conditions, the retention times of compounds [I] and [II] were approximately 4.7 ($k' = 2.0$) and 6.7 min ($k' = 3.3$), respectively. The injection of 0.05 μg of [I] and 0.25 μg of [II] per 10 μl yielded peaks of nearly 50% full scale response, respectively, at a detector sensitivity of $1 \cdot 10^{-2}$ a.u.f.s. The chart speed on the 10-mV Hewlett-Packard, Model 7132A strip chart recorder was 0.635 cm/min.

Standard solutions

Plasma. Weigh out 10.00 mg of compound [I] ($C_{15}H_{23}N_3O_3S$, mol. wt. = 325.43, m.p. = 146°C with decomposition) into a 10-ml volumetric flask and dissolve in 10 ml water. This stock solution containing 1.0 mg [I] per ml (stock solution A) should be prepared fresh daily. Weigh out 10.00 mg of compound [II] internal standard (azidocillin potassium salt; potassium-6-[D-(−)-α-azidophenyl-acetamido]-penicillanate, $C_{16}H_{16}N_5O_4SK$, mol. wt. = 413.5, m.p. = 194°C with decomposition; supplied by Beecham Pharmaceuticals, Chemotherapy Research Center, Betchworth, Great Britain), into a 10-ml volumetric flask and dissolve in 10 ml of water. This stock solution contains 1.00 mg [II] per ml (stock solution D). These stock solutions are used to prepare 10-ml working intermediate solutions B and C containing 100 μg/ml and 10 μg/ml of compound [I], and E containing 100 μg/ml of compound [II], respectively, in water. Aliquots of 10, 20 μl of C, 5, 10, 20 μl of B, and 5, 10, 20 μl of A, respectively, and 25 μl of E (equivalent to 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 or 20.0 μg of [I] and 2.5 μg of [II]) are added to 0.2 ml of control plasma to establish a standard calibration curve for the direct quantitation of the concentration of [I] in the unknowns and for the determination of percent recovery. Aliquots (25 μl) of solution E are added to each unknown plasma as the internal standard [II] in the analysis.

An external standard is prepared by transferring aliquots of 0.5 ml of working standard B and 2.0 ml of internal standard E into a separate 10-ml volumetric flask and diluting to volume with water to yield a solution containing 5 μg [I] plus 20 μg [II] per ml. A 10-μl aliquot of this solution (equivalent to 0.05 μg of [I] and 0.2 μg of [II]) is injected for calculation of percent recovery in plasma and to verify the performance of the HPLC system.

Urine. Weigh out 50.00 mg of compound [I] into a 10-ml volumetric flask and dissolve in 10 ml of control human urine. This stock solution containing 5.00 mg [I] per ml of urine (solution A′) should be prepared fresh daily. Serial dilutions of stock solution A′ are made in control human urine to yield solutions B′, C′, D′, and E′ containing 1 mg, 0.2 mg, 0.05 mg, and 0.025 mg [I] per ml of urine, respectively. Weigh out 10.00 mg of compound [II] (internal standard) into a 10-ml volumetric flask and dissolve in 10 ml of water to yield a stock solution containing 1.0 mg [II] per ml (solution F′). The above standards are used to establish a calibration curve for the quantitation of the unknowns in urine.

Reagents

All reagents are of analytical grade purity and are prepared in deionized distilled water. Phosphate buffer (1.0 *M*, pH 7.0) is prepared by mixing 390 ml 1 *M* KH_2PO_4 (136.1 g/l) and 610 ml $K_2HPO_4 \cdot 3H_2O$ (228.2 g/l). Mix well and adjust to pH 7 with 1 *M* phosphoric acid as needed. Other reagents used include diethyl ether, anhydrous (Mallinckrodt, St. Louis, MO, U.S.A.), acetonitrile and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The mobile phase is filtered through a Millipore filter, type HA, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.) and deaerated prior to use.

Sample handling

Due to the reported instability of [I] in frozen plasma at −25°C [11] it is imperative that the blood specimens be spun down immediately after collection to separate the plasma. The plasma must be stored at 0°C (in an ice bath) if the analysis is to be performed that day (i.e., within 12 h after collection), otherwise the plasma samples must be stored at −70°C (Revco Ultra low Temperature Freezer, Model 800, Rheem Refrigeration Products) to minimize degradation [9]. The urine samples are stored under identical conditions, also due to the reported instability of [I] in the urine at −17 to −20°C [9,10]. The plasma and urine samples stored at −70°C were thawed gradually by keeping the samples in an ice bath with repeated mixing on a vortex-mixer prior to assay. If the volume of the urine sample was greater than 20 ml, the sample was immersed in water (20—25°C) until thawed [10].

Assay procedure

Plasma. Into a 15-ml centrifuge tube, add 0.2 ml of the unknown plasma, 25 μl of internal standard solution E (2.5 μg of [III]) and 0.4 ml of acetonitrile to deproteinate the plasma. Mix for 10—20 sec on a vortex-mixer (Vortex-Genie; ACE Scientific Supply, Linden, NJ, U.S.A.) and then centrifuge the sample in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC Corporation, Needham, MA, U.S.A.) at 0—5°C for 5 min at approximately 2400 rpm (1400 *g*). Transfer the supernatant with a disposable pasteur pipet into a clean tapered 15-ml stoppered centrifuge tube (PTFE No. 13 stopper), and wash the supernatant with 5 ml of diethyl ether (fresh or less than one week old) by shaking for 5 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.). Centrifuge the sample for 5 min and aspirate off the diethyl ether. Immerse the tube containing the aqueous phase in an ice bath prior to HPLC analysis of a 20-μl aliquot (Fig. 2). Along with the samples, process a 0.2-ml specimen of control plasma and eight 0.2-ml specimens of control plasma to which 10, 20 μl of solution C, 5, 10, or 20 μl of solution B, and 5, 10, 20 μl of solution A, respectively, and 25 μl of solution E to each specimen (equivalent to 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20 μg of compound [I] and 2.5 μg of compound [II] each per 0.2 ml plasma, respectively), are added. These standards are used to establish a calibration curve for the direct quantitation of [I] in the unknowns, and for the determination of percent recovery.

Urine. Into a 15-ml centrifuge tube, add 0.1 ml of unknown urine and 0.2 ml of internal standard F′ (200 μg of [II]). Dilute to 1.0 ml with water, mix well and immerse the tube in an ice bath prior to the HPLC analysis of a 10-μl aliquot. Along with the samples, process 0.1-ml specimens of control urine to which are added 0.1-ml aliquots of each of the standards A′, B′, C′, D′, and E′, and 0.2 ml of solution F′, respectively (equivalent to 500, 100, 20, 5, and 2.5 μg of [I] and 200 μg [II]). Dilute these calibration samples to 1.0 ml with water, mix well and immerse the tubes in an ice bath. A 10-μl aliquot of each of these samples is injected to establish a calibration curve for the direct quantitation of the concentration of [I] in the unknowns (Fig. 3).

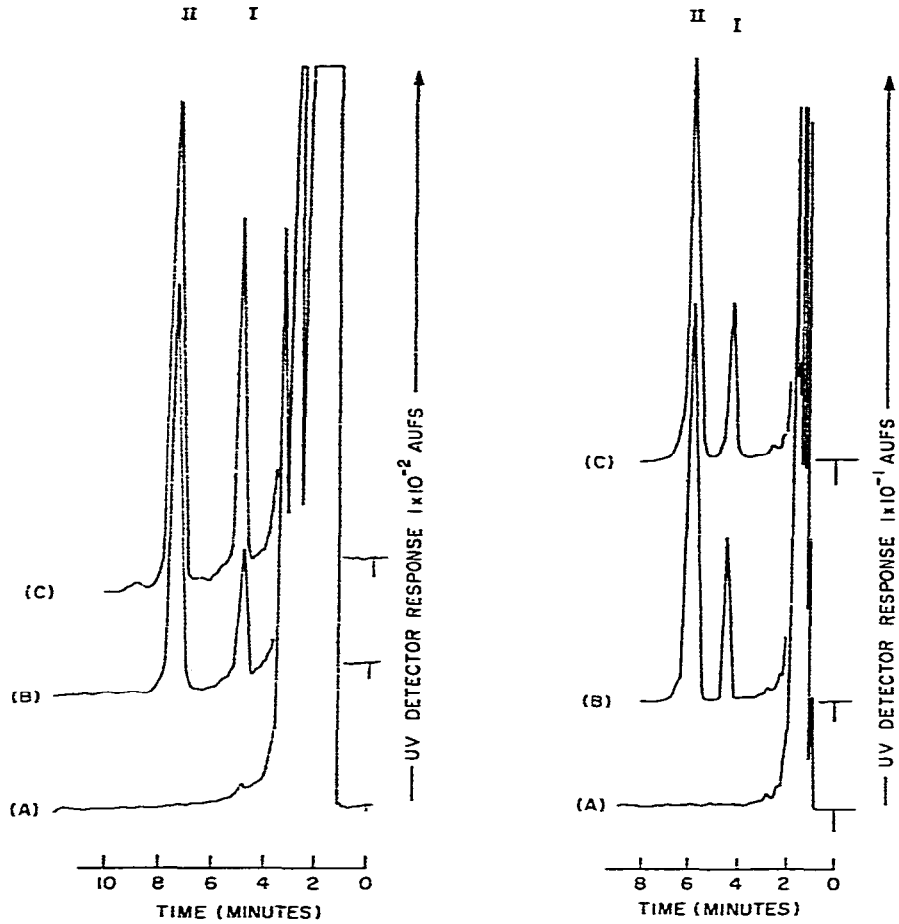


Fig. 2. Chromatograms of HPLC analysis of plasma supernatants of (A) control plasma, (B) control plasma containing 0.5 μg [I] and 2.5 μg [II] per 0.2 ml added authentic standards, and (C) patient plasma 10 min post 10 mg/kg intravenous dose of [I]. Column, Waters, 10 μm μBondapak phenyl; mobile phase, water—methanol—1 *M* phosphate buffer, pH 7 (68:32:0.5) at a flow-rate of 2 ml/min.

Fig. 3. Chromatograms of HPLC analysis of (A) control human urine, (B) control urine containing 0.2 mg [I] per ml and 0.2 mg [II] per ml added authentic standards, and (C) patient urine 4—6 h post 10 mg/kg intravenous dose of [I]. Column, Waters, 10 μm μBondapak phenyl; mobile phase, water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.5) at a flow-rate of 2 ml/min.

Calculations

The concentration of [I] in μg/ml of plasma or mg/ml of urine in each respective unknown is determined by interpolation from calibration curves of internal standards processed along with the unknowns using the peak height ratio (peak height of [I] to peak height of [II] vs. concentration of [I]). The percent recovery of [I] in plasma is calculated from the ratio of peak height of [I] for the external standard and processed standard (assuming the final volume of aqueous phase after diethyl ether wash to be equal to 150 μl).

RESULTS

Statistical validation of the method in plasma and urine

Plasma. The intra-assay linearity and precision of the method was evaluated in plasma over a concentration range of 0.5—100 μg [I] per ml. Triplicate samples at each concentration in the range of 0.1—20.0 μg of compound [I] were added to 0.2 ml of plasma, and taken through the analytical procedure. The data shown in Table I are best described by a power series equation of the form $y = A\ X^B$, ($y = 0.246\ X^{0.977}$) with a correlation coefficient (r) of 0.9996 indicating the high degree of linearity of the method. The method demonstrated excellent precision over the concentration range investigated with mean coefficient of variation of 1.5%. The percent recovery of compound [I] from the plasma was 74.4 ± 6.3% (S.D.) and the sensitivity limit was 0.5 μg [I] per ml.

Inter-assay assay linearity and precision data for the method were accumulated from the clinical studies. The data showed a mean coefficient of variation of 3.5% (Table II).

Urine. The intra-assay linearity and precision of the method were evaluated

TABLE I

LINEARITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY OF COMPOUND [I] IN PLASMA

Compound [I] concentration added (μg/ml)	Mean concentration found ± S.D. ($n = 3$)	Coefficient of variation (%)	Recovery* (%)
0.50	0.53 ± 0.00	0.1	81.3
1.00	0.98 ± 0.02	2.2	79.3
2.50	2.55 ± 0.08	3.3	85.8
5.00	4.94 ± 0.13	2.7	72.1
10.00	9.88 ± 0.05	0.5	67.2
25.00	24.63 ± 0.41	1.7	70.2
50.00	50.50 ± 0.19	0.4	69.4
100.00	105.51 ± 0.94	0.9	70.2
Mean ± S.D.		1.5	74.4 ± 6.3

*The recovery at each concentration represents the mean of three replicate analyses.

TABLE II

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [I] IN PLASMA

Concentration added (μg/ml)	n	Mean concentration found ± S.D.	Coefficient of variation (%)
0.50	4	0.53 ± 0.01	2.3
1.00	4	0.97 ± 0.03	2.7
2.50	4	2.43 ± 0.13	5.3
5.00	4	5.08 ± 0.23	4.6
10.00	5	9.73 ± 0.38	3.9
25.00	4	24.17 ± 1.37	5.7
50.00	4	51.08 ± 1.43	2.8
100.00	4	103.45 ± 1.14	1.1
Mean			3.5

TABLE III

LINEARITY AND PRECISION OF THE HPLC ASSAY OF COMPOUND [I] IN URINE

	Compound [I] concentration added (mg/ml)	*n*	Mean concentration found ± S.D.	Coefficient of variation (%)
Intra-assay	0.025	3	0.024 ± 0.001	3.3
	0.050	3	0.052 ± 0.002	3.9
	0.200	3	0.209 ± 0.005	2.4
	1.000	3	1.005 ± 0.041	4.1
	5.000	3	5.020 ± 0.030	0.6
	Mean			2.8
Inter-assay	0.025	2	0.024 ± 0.001	3.1
	0.050	3	0.049 ± 0.001	2.7
	0.200	2	0.198 ± 0.006	3.2
	0.500	2	0.501 ± 0.006	0.1
	1.000	2	0.965 ± 0.006	0.1
	5.000	3	5.040 ± 0.050	1.1
	Mean			1.7

in urine, using direct injection of triplicate samples of diluted urine in the concentration range of 0.025—5.0 mg [I] per ml. The data are again best described by a power series equation of the form $y = A\ X^B$ ($y = 2.10\ X^{0.973}$), with a correlation coefficient (r) of 0.9997 and a mean coefficient of variation of 2.8% (Table III) with a sensitivity limit of 25 μg/ml.

Inter-assay linearity and precision data for the method were accumulated from the clinical studies. The data showed a mean coefficient of variation of 1.7% (Table III).

Stability evaluation of [I] in plasma

The maximum stability of [I] in aqueous solution has been reported to be at pH 4.5—5.5 [1]. The compound was reported to be unstable in frozen plasma at −25°C, although stability of [I] for a period of three weeks was apparently attained if the acetonitrile protein-free filtrates of plasma were stored at −25°C [11]. Other investigators reported only minimum degradation of [I] in plasma if the sample were stored at −70°C [9]. In this study, the stability of [I] in plasma was evaluated at three concentrations following storage at −70°C for a period of 50 days.

A stock pooled sample was prepared by adding three 100-μl aliquots of stock solution A (containing 1.0 mg [I] per ml) to three separate 9.9-ml aliquots of fresh control human plasma, mixing well, and combining (total 30.0 ml) to yield a pooled stock containing 10 μg [I] per ml. This pooled plasma stock sample was used to prepare two other pooled samples containing 7.5 and 5 μg [I] per ml, by transferring 7.5 and 5 ml of the stock pool A, and diluting to 10 ml with control (drug-free) human plasma. These three pooled plasma samples were then subdivided into 1-ml aliquots in 15-ml polypropylene tubes and stored at −70°C for a specific time interval prior to analysis.

TABLE IV

STABILITY OF [I] IN PLASMA STORED AT −70°C

All assays were performed on triplicate samples (n = 3).

Day	Amount found (μg) (Amount added = 5.00 μg)	Amount found (μg) (Amount added = 7.50 μg)	Amount found (μg) (Amount added = 10.00 μg)
0	5.08 ± 0.16	7.60 ± 0.16	10.36 ± 0.13
6	5.05 ± 0.34	8.07 ± 0.07	10.44 ± 0.29
21	4.86 ± 0.22	7.48 ± 0.30	9.83 ± 0.29
33	5.49 ± 0.36	8.14 ± 0.07	10.43 ± 0.25
50	5.72 ± 0.04	8.32 ± 0.22	10.59 ± 0.38

TABLE V

PLASMA CONCENTRATION (μg/ml) OF TWO HUMAN SUBJECTS FOLLOWING INTRAVENOUS ADMINISTRATION OF A 10 mg/kg DOSE OF [I] MEASURED BY HPLC AND MICROBIOLOGICAL ASSAY

Sample time	Subject I		Subject 2	
	HPLC	Microbiological assay [13]	HPLC	Microbiological assay [13]
Infusion + 5 min	42.2	42.9	4.2	3.3
Infusion + 10 min	55.6	60.4	6.5	5.9
0 h	38.1	37.3	79.2	86.0
5 min	N.S.*	N.S.	34.6	34.3
10 min	N.S.	N.S.	27.5	30.2
15 min	39.0	36.3	22.0	22.2
30 min	24.5	24.3	16.3	15.4
1 h	13.7	10.0	7.9	6.8
1.5 h	7.7	3.9	4.4	3.5
2 h	N.S.	3.2	2.3	1.9
3 h	1.4	1.4	N.S.	0.9
4 h	0.5	0.7	N.S.	0.6
6 h	N.S.	N.S.	0.4	0.4
HPLC vs. microbiological		r = 0.994 (n = 9)		r = 0.999 (n = 11)

*N.S. = no specimen.

Aliquots (0.2 ml) of each of the pooled samples were analyzed in triplicate by the described procedure on the days indicated in Table IV. The data (Table IV) for the stability of [I] in plasma (physiological pH of ca. 7.4) stored at −70°C showed no discernible degradation over a 50-day period. The data indicate that plasma specimens must be frozen at −70°C immediately on collection and remain at that temperature until analyzed. Any deviation from these storage conditions will result in significant degradation of [I] and consequently invalidate any data generated from these samples.

Application of the method to biological specimens

Plasma concentrations of [I] were measured by HPLC in two human subjects following intravenous doses of 10 mg [I] per kg body weight. The data (Table V) show an excellent correlation of the HPLC assay with the microbiological procedure [13] for the two sets of samples (r = 0.994 and 0.999) demonstrating that the two procedures are equivalent.

The urinary excretion profiles of [I] were also measured by HPLC in the same subjects. The percent of dose recovered as unchanged [I] in the 0—24 h excretion period accounted for 66.6% and was in good agreement with data previously reported using a microbiological assay [3]. A good correlation (r = 0.986) was obtained for the determination of the concentrations of [I] by the HPLC and microbiological assays.

DISCUSSION

The high polarity of highly ionized amphoteric zwitterionic compounds such as [I] precludes their direct extraction from biological fluids into an organic solvent, consequently analysis can only be performed following the formation of a protein-free filtrate of plasma or a dilution of urine. The analysis of these aqueous samples is only possible with reversed-phase HPLC. Preliminary experiments utilizing the acetonitrile protein-free filtrate procedure previously described [11], showed that soluble endogenous compounds derived from the plasma sample often interfere with the measurement of [I] or [II]. In addition, the stability of [I] under the conditions of evaporation was questionable.

The introduction of the diethyl ether wash of the protein-free filtrate effectively removes the interfering endogenous compounds and also extracts the acetonitrile to leave an aqueous phase of approximately 0.15 ml which is suitable for direct injection. This wash step also eliminated the problematic and time-consuming evaporation step. The concentrations of drug present in the urine is well in excess of 20 μg/ml and thus no interference from endogenous compounds is noted.

Azidocillin (potassium salt), [II], was selected as the internal standard for the analysis of [I] in plasma and urine due to its chemical stability and compatible chromatographic properties. Two other antibiotics, penicillin V [III], and propicillin [IV], and the hypnotic methyprylon [V] (Fig. 1) were also found to be suitable for use as internal standards for the assay of [I] with minor changes in the mobile phase composition (Table VI). Compound [III] which showed excellent chromatographic behaviour (Fig. 4) was not selected as the internal standard due to the high ionic strength (0.01 M) phosphate buffer required, which showed deleterious effects on column performance over long periods of use. During the initial stages of development, propicillin [IV] was used very successfully as the internal standard. However, the disadvantage using

TABLE VI

INTERNAL STANDARDS FOR THE ANALYSIS OF COMPOUND [I]

Compound	Composition of mobile phase, water—methanol—1 M phosphate buffer, pH 7	Flow-rate (ml/min)	k'	k' [I]
II, Azidocillin (potassium salt)	70:30:0.5	2.0	3.3	2.0
III, Penicillin V	65:35:1.0	1.5	2.4	1.6
IV, Propicillin	70:30:0.1	2.0	3, 3.4	1.8
V, Methyprylon	70:30:0.1	2.0	2.6	1.8

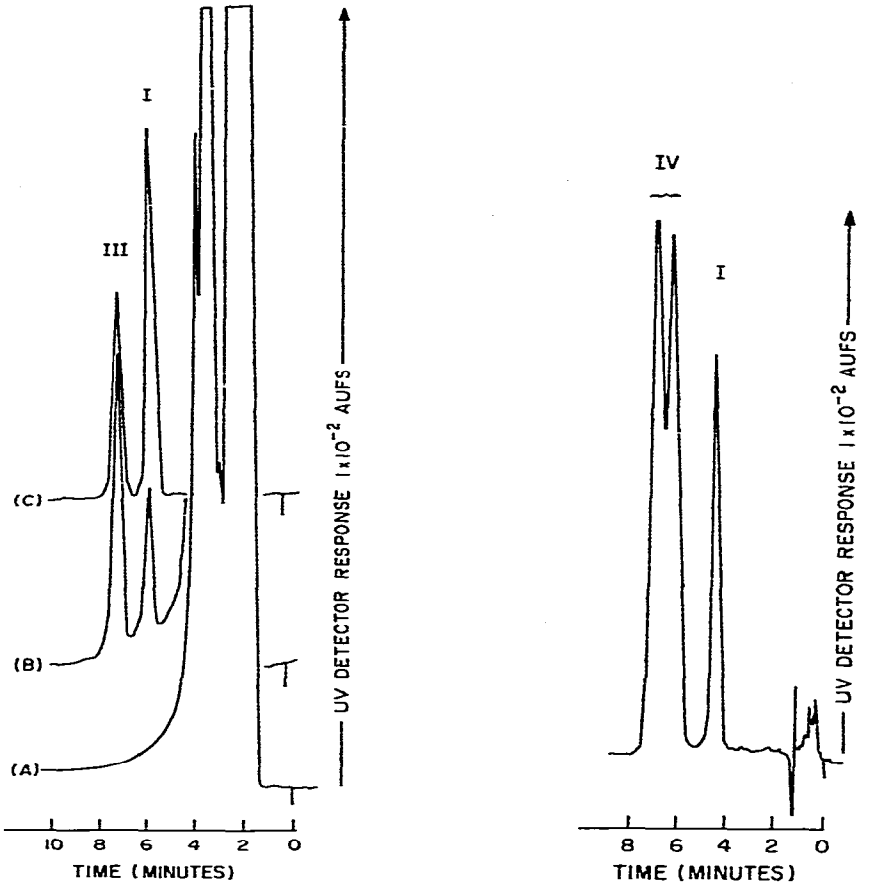


Fig. 4. Chromatograms of HPLC analysis of plasma supernatants of (A) control plasma, (B) control plasma containing 0.5 μg [I] and 25 μg penicillin V [III] per 0.2 ml added authentic standards, and (C) 100 ng [I] and 1000 ng [III] authentic standards. Column, Waters, 10 μm μBondapak phenyl; mobile phase, water—methanol—1 *M* phosphate buffer, pH 7 (65:35:1.0) at a flow-rate of 1.5 ml/min.

Fig. 5. Chromatogram of HPLC analysis of 100 ng authentic standard [I] and 100 ng propicillin [IV] on a μBondapak phenyl column and mobile phase of water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.1) at a flow-rate 2 ml/min.

this particular penicillin is the presence of two distinct peaks due to the diastereoisomers in the analytical standard (Table VI, Fig. 5), which complicated the measurement of peak height of this compound. The average peak height ratio of the two isomer peaks is 1.04 which is in good agreement with phase solubility data which indicated that the L-isomer was present to the extent of 57% [14]. An exact correlation with phase solubility is difficult due to lack of information pertaining to the ratio of UV absorptivities of the two isomers. Methyprylon [V] is an excellent internal standard (Table VI) for the analysis of urinary [I] and has been used in several preliminary investigations. However, since the compound is extracted into the diethyl ether wash of the protein-free filtrate of plasma, it cannot be used in the plasma assay.

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