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Note

Determination of mecillinam in urine by reversed-phase high-performance liquid chromatography

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Pivmecillinam hydrochloride (I), the pivoloyloxymethyl ester of mecillinam, and mecillinam (II) (Table I) are 6-amidinopenicillanic acid derivatives undergoing clinical evaluation as antibiotics effective against gram-negative organisms [1]. Pivmecillinam hydrochloride was specifically developed as an orally active form of mecillinam. It is a prodrug which is well absorbed from the gastrointestinal tract, and whose activity is due to its rapid biotransformation to mecillinam, the active antibiotic agent [2]. Mecillinam, however, is administered only parenterally. The synergistic activity of mecillinam with other β-lactam antibiotics is of significant clinical importance in chemotherapy [3].

The determination of mecillinam in biological fluids (plasma and urine) is performed mainly by microbiological assay methods [4], by which valuable pharmacokinetic and biopharmaceutic information in humans has been obtained [5—8]. However, these procedures require special laboratory conditions for their routine use, and a more generally applicable chemical assay was therefore sought for clinical pharmacokinetic studies.

A spectrophotometric method involving the formation of a 4-aminomethyleneimidazol-5-(4*H*)-one derivative which was measured at 330 nm, was published for the determination of mecillinam and used for the study of the degradation kinetics of the drug [9, 10]. This principle was also used for the determination of the compound in plasma or urine by high-performance liquid chromatographic analysis via post-column derivatization [10].

As mecillinam is thermally unstable and non-extractable from aqueous media owing to its amphoteric nature, analysis by high-performance liquid chromatography (HPLC) is a very practicable means for its chemical analysis. The technique has been used successfully in the analysis of several types of antibiotics, viz., kanamycin [11], tetracycline [12], gentamycin [13],

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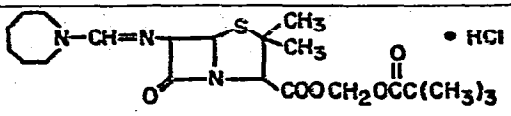
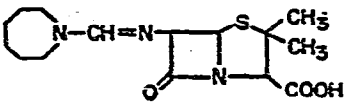
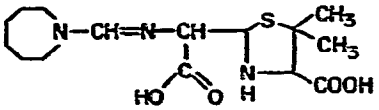
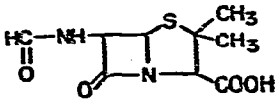
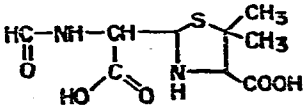
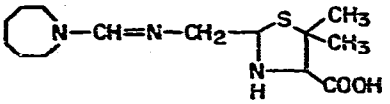
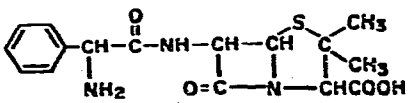
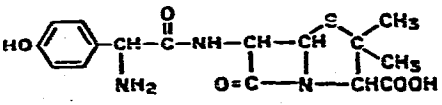
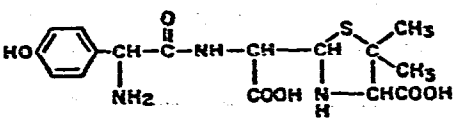
TABLE I

STRUCTURES AND CAPACITY FACTORS (k') OF PIVMECILLINAM HYDROCHLORIDE AND RELATED COMPOUNDS

Compound	Name	Structure	Retention time (min)	k'
I	Pivmecillinam		>30	—
II	Mecillinam		4.25	2.5
III			1.8	0.5
IV			1.4	<0.5
V			1.4	<0.5
VI			4.5	2.75
Related compounds	Ampicillin		2.2	0.8
	Amoxycillin		1.6	<0.5
	Amoxycillinpenicilloic acid		1.4	<0.5

cephalothin [14], chloramphenicol [15] and amoxicillin [16, 17]. These methods use either the intrinsic UV absorbance of the compound or the fluorescence of a suitable derivative (fluorescamine or *o*-phthalaldehyde), prepared post-column as the means of detection. The intrinsic UV absorbance of mecillinam at 220 nm was used for stability testing of the bulk drug and for quality assurance of its dosage forms by HPLC [18].

The HPLC procedure presented here is an adaptation of a previous method [18] and involves dilution of a urine specimen followed by reversed-phase HPLC analysis and detection of intact mecillinam by its UV absorbance at 220 nm. As the drug is extensively eliminated in urine (60% of a dose eliminated in a 12 h excretion period), determined by microbiological assays [5—8], its concentration in urine is sufficiently high to be determined by direct analysis of a dilution of urine. The HPLC assay has a sensitivity limit of 0.05 mg/ml (50 μg/ml) using a 1-ml urine sample per assay, equivalent to a minimum detectable amount of 50 ng of mecillinam injected on to the column.

EXPERIMENTAL

Column

A pre-packed, 30 cm × 4.6 mm I.D. stainless-steel column containing a 10-μm Chromegabond C_{18} reversed-phase microparticulate packing (Serial No. 289-1-29-821, E.S. Industries, Marlton, N.J., U.S.A.) was used.

Instrumental parameters

A Waters Model 6000A high-pressure liquid chromatography pump, equipped with a Model U6K injection system and a Waters pre-column filter (2 μm) (Waters Assoc., Milford, Mass., U.S.A.), was used for chromatography. A Tracor Model 970A variable-wavelength absorbance detector (Tracor Instruments, Austin, Texas, U.S.A.) was used for quantitation at 220 nm.

The isocratic mobile phase consisted of 15% acetonitrile (UV grade) in 0.01 *M* potassium buffer (pH 5.0) pumped at a constant flow-rate of 2.0 ml/min. Under these conditions the retention time of mecillinam was 4.25—4.5 min (Fig. 1). The chart speed of the Hewlett-Packard dual-channel recorder (Model 713A with option 108) was 0.5 in./min.

Reagents

All reagents were of analytical-reagent grade (A.C.S.). All aqueous buffers were prepared in distilled, carbon-filtered, deionized water filtered through a 0.2-μm filter (Type DC System, Hydro Service and Supplies, Durham, N.C., U.S.A.).

Potassium phosphate buffer, 0.01 *M* (pH 5.0), was prepared by titration of 0.01 *M* KH_2PO_4 (1.361 g/l) with a small amount of 0.01 *M* K_2HPO_4 (anhydrous) (1.742 g/l) until the desired pH was attained.

Acetonitrile (UV grade), "distilled in glass" and suitable for both spectrophotometry and liquid chromatography, was purchased from Burdick & Jackson, Muskegon, Mich., U.S.A.

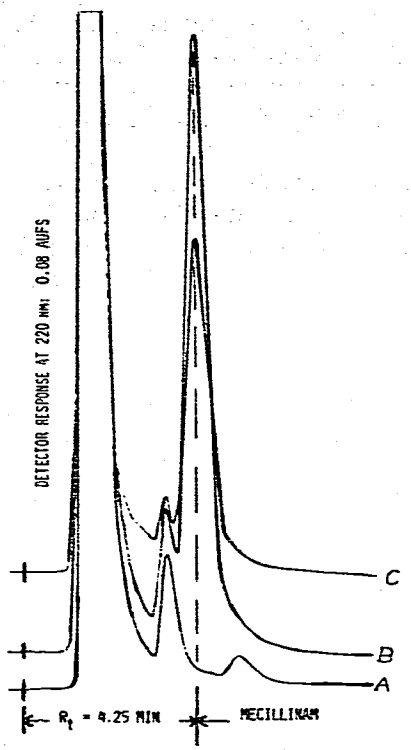


Fig. 1. Chromatograms of (A) control human urine, (B) control urine containing 2 mg/ml of authentic mecillinam added and (C) patient urine 4—6 h after a 15 mg/kg intramuscular dose.

Analytical standard solutions

Stock solutions of mecillinam, 6-β-{[(hexahydro-1*H*-azepin-1-yl)-methylene]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3.2.0)heptane-2-carboxylic acid, $C_{15}H_{23}N_3O_3S$, mol. wt. 325.41, m.p. 146° (with decomposition), 99% purity, were prepared on each day of analysis in fresh control human urine. Fifty milligrams of mecillinam were weighed into a 10-ml volumetric flask and dissolved in and diluted to volume with control human urine to yield a stock solution A containing 5 mg/ml. Serial dilutions of solution A were made in urine to yield standard solution B containing 1 mg/ml, solution C containing 0.2 mg/ml and solution D containing 0.05 mg/ml.

Note. All standard solutions must be made up fresh daily and discarded after use. The solutions are stable over a 24-h period at 4° (refrigerator temperature) but are unstable over a 48-h period.

Procedure

The urine specimens to be analyzed were taken from a freezer at —70° and allowed to thaw at room temperature while the working standard solutions were being prepared. If the volume of the urine sample was greater than approximately 10 ml, then the containers were partially immersed in water (20—25°) to speed up the thawing. The thawed urine specimens, together

with standard solutions A—D and control urine (E), were diluted from 1.0 ml to 10.0 ml with water in volumetric flasks and mixed well. If a diluted urine sample appeared cloudy or had a visible sediment then a 1.0-ml aliquot was filtered through a 0.45-μm pore size Millipore membrane filter for clarification.

A 10-μl aliquot of each of the clear diluted urine samples, standards A—D and control urine (E) was injected for HPLC analysis. The standard solutions A—D are used to establish a concentration—response curve for the calculation of the amount of mecillinam in unknown samples for each day of analysis.

Calculations

The concentration of mecillinam in unknown samples is determined by interpolation from the calibration graph for the working standard solutions (A—D) in the concentration range 0.05—5.0 mg/ml, processed together with the unknown samples, using the direct calibration (peak height versus concentration) technique. The same working standard solutions are also used to establish the chromatographic retention volume, and the sensitivity and linearity of the detection system for each day of analysis.

RESULTS AND DISCUSSION

Plasma levels of mecillinam determined by microbiological assay methods [4] are relatively low (C_p^{max} <10 μg/ml) following single 200-mg i.v. doses [6] owing to its rapid elimination in the urine. The concentration of the drug in urine, however, is sufficiently high [5—8] to be determined directly in diluted urine by HPLC using UV detection at 220 nm. The relatively weak UV absorbance of the compound limits the sensitivity of the method. Sample dilution is essential to reduce the introduction of endogenous particulate material onto the column, which would otherwise impair its performance. The high water solubility and amphoteric nature of mecillinam necessitates the use of reversed-phase HPLC for its analysis. The intra-assay relative standard deviation with 0.20—5.0 mg of mecillinam added per millilitre of urine is 1.5% (Table IIA), whereas the inter-assay relative standard deviation over the same concentration range is 6.3% (Table IIB).

Sample storage and handling

Studies on the kinetics and mechanism of the degradation of mecillinam in aqueous solutions [10] have demonstrated that for optimal stability, the pH should be maintained at 4.5—6.0, i.e., at about the isoelectric pH for this amphoteric drug. The acid stability of mecillinam is comparable to that of ampicillin, one of the most acid-stable penicillins. However, at neutral and weakly basic pH, mecillinam is 5—10 times more susceptible to hydrolysis of the β-lactam ring to penicilloic acid than is ampicillin [10]. Hence the stability of mecillinam at physiological pH becomes critical and can be assured only by storage at low temperature.

Mecillinam is unstable in urine even at freezer temperatures (—17 to —20°) (Table IIIA), the mean recovery declining from 88% at 16 days to 80% at

TABLE II

STATISTICAL ANALYSIS OF ASSAY PARAMETERS

Parameter	Mecillinam added (mg/ml)	Replicates (n)	Mean response (absorbance units × 10^{-4})	Mecillinam found (mg/ml)	Relative standard deviation (%)
(A) Intra-assay precision*	0.00	2	12	0.015	—
	0.05**	4	36	0.051	7.8
	0.20	4	124	0.194	1.3
	1.00	4	620	0.987	1.8
	5.00	4	3140	5.000	1.5
(B) Inter-assay reproducibility	0.05**	3	—	0.047	14.5
	0.20	6	—	0.184	8.5
	1.00	6	—	1.020	6.3
	5.00	6	—	5.090	4.2

*Calculated from least-squares curve fit. Linear least-squares regression analysis: $y = mx + b$. Slope = 1.565, intercept = +0.0065. Correlation coefficient (r) = 0.9999.
**Limit of detection.

40 days. Although storage in dry-ice for shipment is satisfactory for short-term stability (48 h), long-term stability (Table IIIB) requires storage at temperatures below —70° (e.g., Revco Ultra Low Temperature Freezer, Series/Models 800 or 1000, Rheem Refrigeration Products Div., S.C., U.S.A.).

Samples stored at —70° should be allowed to thaw gradually to room temperature, without exposure to elevated temperatures, and analyzed expeditiously, i.e., within 4 h of thawing to room temperature.

Application of the method to biological fluids

Urine specimens were collected at various time intervals over a 24-h excretion period in three subjects following a single 15 mg/kg intravenous or intramuscular administration of mecillinam (Table IV). The rapid elimination of intact mecillinam is indicated by the extensive recovery of the dose over the 0—6 h excretion interval. The data on urinary excretion obtained with the HPLC assay are in good agreement with those previously reported using microbiological assays following either the parenteral administration of mecillinam [5—8] or the oral administration of pivmecillinam [19—21].

Specificity of the assay

Pivmecillinam hydrochloride (I) when administered orally is rapidly biotransformed by de-esterification into mecillinam (II) (the active antibiotic). No measurable plasma levels of I have yet been reported [5, 21]. Under the HPLC conditions used in this assay, I is not eluted from the column even after 30 min, and it would require a more polar mobile phase for elution [22] than is described here; hence it does not interfere with the analysis of mecillinam.

TABLE III

STABILITY OF MECILLINAM IN URINE ADDED AT CONCENTRATIONS RANGING FROM 0.2 TO 5.0 mg/ml AT DIFFERENT TEMPERATURES

(A) Stability at −17°

Storage time (days)	Mean concentration found (%)	Standard deviation (%)
16	88	4.8
30	86	3.3
40	80	3.2

(B) Stability at −70°

Storage time (days)	Concentration added (mg/ml)	Concentration found (mg/ml)	Recovery (%)	Mean recovery (%)	Standard deviation (%)
10	0.2	0.21	107.0	101.5	4.7
	1.0	0.99	98.8		
	5.0	4.99	98.8		
20	0.2	0.19	98.5	94.8	11.3
	0.2	0.15	72.5		
	1.0	0.97	97.0		
	1.0	1.05	105.0		
	5.0	4.96	99.2		
	5.0	4.83	96.6		
	0.2	0.17	85.0	102.9	16.6
	0.2	0.17	86.0		
	1.0	1.08	108.0		
	1.0	1.21	121.0		
	5.0	4.77	95.4		
	5.0	6.10	122.0		
40	0.2	0.18	90.0	97.2	8.2
	0.2	0.18	92.0		
	1.0	0.98	98.3		
	1.0	0.98	98.0		
	5.0	5.29	105.8		
	5.0	4.94	98.8		
50	0.2	0.18	92.0	97.6	5.9
	1.0	0.99	99.0		
	5.0	5.01	100.2		
			Overall mean:	98.8	9.4

Several urinary metabolites of pivmecillinam and mecillinam have been reported [6, 21] (Table I). Of these, compound III, the penicilloic acid of mecillinam, and compound VI, a metabolite identified only in the rat [6, 21], have the same azomethine (C = N) chromophore as mecillinam. Compound III has a retention time of 1.8 min and is completely resolved from

TABLE IV

URINARY EXCRETION PROFILE IN HUMANS FOLLOWING A SINGLE 15 mg/kg INTRAVENOUS OR INTRAMUSCULAR DOSE OF MECILLINAM

Collection interval (h)	Patient (initials; sex; weight; dose; route)								
	L.W.; m; 77 kg; 1155 mg; i.m.			G.K.; m; 84 kg; 1260 mg; i.v.			F.K.; f; 62 kg; 930 mg; i.v.		
	Concentration (mg/ml)	Total excreted (mg)	% of dose excreted	Concentration (mg/ml)	Total excreted (mg)	% of dose excreted	Concetration (mg/ml)	Total excreted (mg)	% of dose excreted
−2 to 0	nm	—	—	nm	—	—	nm	—	—
0–1	1.79	71.6	6.2	—*	—	—	2.15	236	25.4
1–2	—*	—	—	1.07	139	11.0	—*	—	—
2–4	4.28	428.0	37.1	—*	—	—	2.32	220	23.7
4–6	0.07	12.6	1.1	2.88	262	20.8	0.54	54	5.8
6–8	nm	—	—	0.12	5.4	0.4	—*	—	—
8–12	nm	—	—	0.04	1.8	0.1	0.05	8	0.9
12–24	—	—	—	nm	—	—	nm	—	—
Total excreted		512.2	44.4		408.2	32.4		518	55.7

*Subject did not void at the end of the excretion period. Subsequent sample collected represents a "combined" pool for the two intervals.

mecillinam (retention time 4.25 min). It is a measurable urinary metabolite in man and accounted for about 6% of an oral dose of pivmecillinam [21]. The capacity factors for a number of metabolites common to pivmecillinam are also listed in Table I. Compounds IV and V are minor metabolites, accounting for 2—6% of an oral dose. They do not interfere, as they are eluted in the solvent front and are also weaker chromophores than mecillinam. Other related antibiotics which are co-adminstered with mecillinam, such as ampicillin and amoxicillin (and their respective penicilloic acids), which could be sources of interference, are all resolved from mecillinam, ensuring the specificity of the assay in urine.

The capacity factors of a number of compounds characterized as breakdown products and/or metabolites of pivmecillinam/mecillinam, using mobile phases analogous to that used on this study, have been reported by Hagel and Waysek [22] as a stability indicating reversed-phase HPLC assay for pivmecillinam hydrochloride in a capsule dosage form, and in susceptibility disks [23]. All of these breakdown products, which should not be present in frozen urine samples (—70°), would be eluted in the solvent front owing to the small k' values obtained with the mobile phase used in this study, and hence would not interfere with the specificity of the assay for mecillinam.

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