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STABILITY-INDICATING ASSAY FOR MECILLINAM USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-pressure liquid chromatographic method has been developed for use as a stability-indicating assay for mecillinam. Seven related compounds and at least one unknown degradation product are well separated and easily determined. The accuracy of the method is in good agreement with the published UV assay and the precision of a series of seven replicate determinations is ±1.0% R.S.D.

INTRODUCTION

The analysis of penicillin-type antibiotics by various chemical and biological methods has been well documented in the literature since their introduction in the 1940's. While references to the analysis of these compounds are too numerous to quote, an excellent overview of this subject is presented by Hughes *et al.*[1], in a review article published in 1976.

With the development and widespread use of high-pressure liquid chromatography (HPLC), one sees an increasing number of publications describing the analysis of penicillins and other antibiotics with special emphasis on the bonded-phase chromatography of these compounds[2–5]. In an effort to develop a rapid, stability-indicating assay for mecillinam, we have investigated the HPLC properties of this and related compounds.

Mecillinam, a β-lactam antibiotic developed by Leo Pharmaceuticals (Ballerup, Denmark), is one of a new class of amidino penicillins, characterized by an hexahydroazepine-substituted amidino group in the 6-position rather than a 6-β-acylamino derivative. Mecillinam shows biological activity against gram-negative organisms[6,7] and when tested in combination with acylamino penicillins, also exhibits a synergistic effect against those organisms *in vitro*[8].

Its target site in *Escherichia coli* has been determined to be exclusively penicillin-binding protein 2 (PBP2) as opposed to other penicillins which bind weakly with PBP2 and more strongly with other PBP. This PBP binding seems to disturb

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peptidoglycan metabolism and inhibits cell division and elongation leading, eventually, to cell lysis[9].

Mecillinam (I) and several related compounds, which are known to be degradation products or manufacturing impurities, are described in Table I. The precise mode of degradation has been published[10] and work at Leo Pharmaceuticals has resulted in the positive identification of all the compounds listed in Table I, and authentic samples were synthesized by the Research Division of Roche Laboratories in order to aid in the analytical methods development.

The current chemical assay for mecillinam has been reported by Larsen and Bundgaard[11] and consists of derivatization of mecillinam by formation of a glycine adduct followed by subsequent rearrangement to a chromophore with an absorbance maximum at 330 nm. The 330 nm maximum is specific for the mecillinam derivative; however, the method does not permit estimation of any of the other compounds. In order to analyze these compounds as well as mecillinam, the HPLC methods described in this report were developed.

EXPERIMENTAL

Chemicals and reagents

Mecillinam and related compounds were provided by Leo Pharmaceutical Products Ltd. or by the Organic Chemical Research Department, Hoffmann-La Roche Inc. Purity criteria and structural confirmation consisted of acceptable spectral and chromatographic data derived from standard methods of analysis. All other reagents were A.C.S. reagent grade or equivalent, except that chromatographic grade solvents were used for all chromatography and were passed through a 0.5-μm filter prior to use.

Sample preparation

All of the compounds were dissolved in filtered, distilled water, diluted appropriately and chromatographed without any additional treatment.

Chromatographic equipment

The use in this laboratory of component HPLC systems leads to a variety of instrumental combinations. All of the following components were used interchangeably without problem. Pumps: Model 396 Mini Pump (Laboratory Data Control); Model 6000A Chromatographic Pump (Waters Assoc., Milford, Mass., U.S.A.). Injectors: Model U6K (Waters Assoc.); Model 7120 (Rheodyne Inc.). Detectors: Model 970A variable wavelength (Tracor); Model 1202 variable wavelength (Laboratory Data Control).

30 cm $\times$ 3.9 mm I.D. μBondapak C_{18} columns (Waters Assoc.) were used throughout, and chromatograms were recorded on a 10-in. strip chart recorder. Instrument parameters: eluant flow-rate, 1.0 ml/min; detector wavelength, 220 nm; detector sensitivity, 0.32 absorbance units full scale (a.u.f.s.) for analysis of 0.1 mg/ml sample solutions, or as required to achieve measurable response.

Mobile phase

A series of eight mixtures of acetonitrile–0.01 M sodium phosphate (pH =

TABLE I

STRUCTURES AND CAPACITY FACTORS OF MECILLINAM AND RELATED COMPOUNDS

Compound	*Structure*	k'_A	k'_B
Group 1			
I (Mecillinam)		2.83	
II		7.00	
III		5.00	—
IV		7.92	—
Group 2			
V		<1	3.95
VI		<1	1.63
VII		<1	1.51
VIII		<1	2.43

5.0) ranging from 25:75 to 0:100 respectively were found to be useful for the HPLC of mecillinam. The two systems which effectively separated all the compounds at reasonable times were A (15:85) and B (2.5:97.5).

Thin-layer chromatography

An absorbant of silica gel GF_{254} was used for thin layer analyses. The developing solvent was *n*-butyl acetate–isopropanol–acetic acid–0.07 *M* citrate buffer (pH = 7) (90:60:75:30). Visualization was achieved by exposure to iodine vapor followed by spraying with 1% aqueous starch solution.

RESULTS AND DISCUSSION

The UV spectra of mecillinam and some related compounds appear in Fig. 1. From a quick inspection of these spectra, it is obvious that the UV detection of

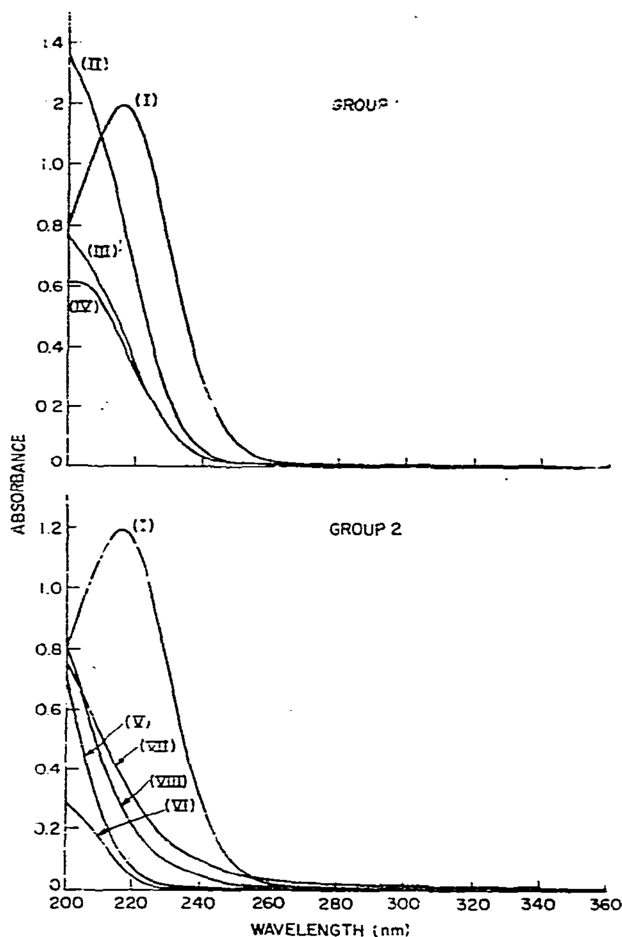


Fig. 1. The UV spectra of mecillinam and related compounds at concentrations of 20 μg/ml. Mecillinam (I) has been included in both groups for reference purposes.

reasonably low levels of these compounds would be restricted to the spectral region 200–230 nm. A test of response *versus* noise for each of the compounds over this range indicated that 220 nm would yield an effective compromise between detection sensitivity and detector noise level.

During the course of development of the methods described in this report, numerous HPLC systems were investigated. Unbuffered acetonitrile–water, methanol–water and dioxane–water systems were found to be unacceptable due to poorly shaped peaks. Tailing on both edges of the peaks indicated the need for a buffered system, and the necessity for working at low wavelengths led to the use of phosphate-buffered mobile phases. The end results of testing a number of acetonitrile–0.01 *M* sodium phosphate buffers were two eluants which were sufficient to separate the various compounds. Thd ratios of acetonitrile:buffer in these eluants were 15:85 (A) and 2.5:97.5 (B).

Retention characteristics

A simple division in Table I into compounds containing a hexahydroazepine ring (I–IV) and those without the ring (V–VIII) gives two groups of entities which exhibit similar chromatographic behavior. The compounds in group 1 are less polar, strongly retained and chromatograph well in mobile phase A; group 2 are more polar, weakly retained and chromatograph well in mobile phase B. In performing assays for individual compounds, the appropriate mobile phase must be selected.

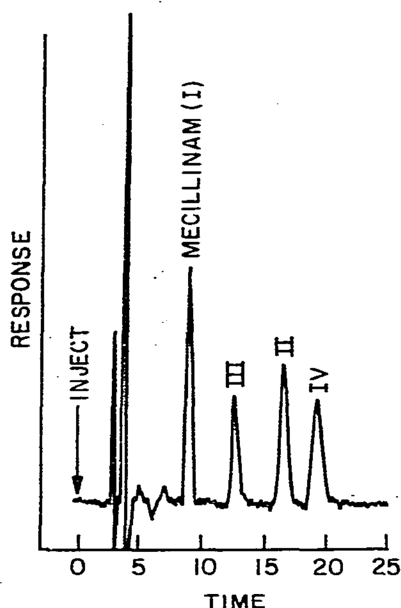
Although the primary object of this study, the assay of mecillinam in the presence of a series of related compounds and the determination of those compounds was satisfied by using systems A and B, the relative retentions of all the compounds studied could be shifted considerably by varying the nonpolar component of the mobile phase, and it is left to the investigator to select the best solvent ratio to suit his exact needs for a particular sample which may contain a variety of excipients or interferences.

The capacity factors for each compound are given in Table I for both eluents A and B. In system A, group 1 compounds are well resolved and differences in capacity factors (k') are one or greater. In system B, group 1 compounds do not elute after 1 h. In system A, group 2 elutes rapidly with k' values all less than one; however, in system B, group 2 is well resolved except for VI and VII. When it is necessary to separate VI and VII, 100% phosphate buffer can be used for total resolution. Sample chromatograms are presented in Figs. 2 and 3 for systems A and B.

Quantitative analysis of mecillinam

Mecillinam currently is targeted for parenteral use as unreconstituted vials of pure drug; therefore, analytical systems which are developed for bulk drug assays can also be applied to the dosage forms without modification.

Using system A, a linear relationship was observed between weight of mecillinam injected and peak height (expressed in mm) for convenient analytical sample weights ranging from 0.500 to 1.500 μg. The parameters for the line $y = mx + b$ are $m =$ 142, $b = 0.327$ and $r = 0.99995$. The limit of detection of mecillinam, defined as the amount required to give a signal greater than twice the noise level, was 2 ng when measured under the most favorable conditions.



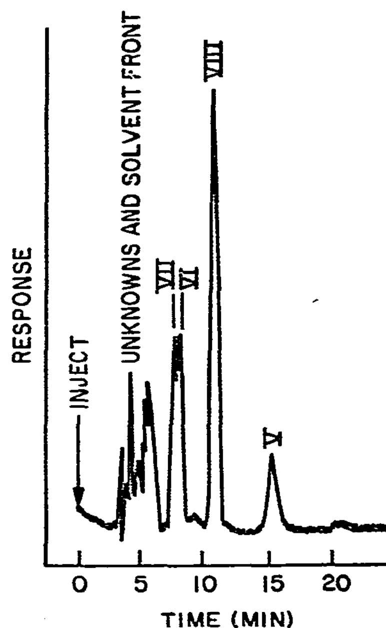


Fig. 2. HPLC chromatogram of group 1 compounds.

Fig. 3. HPLC chromatogram of group 2 compounds.

A series of injections of a single sample was performed to test system reproducibility. The average peak height for seven replicate 1.04-μg injections from a single solution was 109.2 ± 1.1 mm (coefficient of variation = 1%). Finally, a series of replicate assays were performed on six samples of mecillinam parenteral vials. Data for these assays appears in Table II along with results for the glycine derivative UV assay performed simultaneously. Inspection of the data shows excellent correlation between both methods with a maximum difference of 0.9% for any comparative assay.

In order to verify that the HPLC methods provide true stability-indicating assay data, several experiments were performed. First, a sample of mecillinam stan-

TABLE II

COMPARATIVE ASSAY DATA FOR HPLC AND UV DERIVATIVE METHODS

All values are averages of duplicate determinations.

Sample	*mecillinam (%)*	
	HPLC assay	*UV assay*
1	99.0	99.4
2	99.5	99.6
3	99.5	100.0
4	100.9	100.0
5	100.2	99.9
6	100.3	100.9

dard was spiked with compounds II–VIII at the 0.5% level (equivalent to *ca.* 25 ng of each impurity per injection) and chromatographed. Both groups of impurities were well resolved and easily quantitated. A typical analysis for II, III and IV based on peak-height measurements for injections gave assay precisions of 6.1, 4.3 and 5.2% respectively which is acceptable reproducibility for low-level impurity determinations. Next, a sample of pure drug which had been stored at 55° for *ca.* three months was analyzed for compounds II–VIII. In Table III, the data are compared to thin-layer chromatographic analysis of the same material with good correlation.

TABLE III

MILD THERMAL DEGRADATION OF MECILLINAM

nd = None detected.

Compound	*Assay (%)*	
	HPLC	*TLC or UV**
Mecillinam (I)	97.9	97.0*
II	1.56	1.0
III	0.67	0.4
IV	trace	0.3
V	nd	nd
VI	nd	nd
VII	nd	nd
VIII	nd	nd

As a further test of the analytical versatility of HPLC systems A and B, pure mecillinam was degraded enzymatically by reaction with penicillinase derived from *Bacillus cereus*. The expected product of this reaction is the mecillinam penicilloic acid formed by cleavage of the β-lactam ring. Chromatography of the solution showed the formation, and increase with time, of a peak at $k'_A = 1.08$, $k'_B = 5.0$. This peak is separated from all the known compounds and in the absence of an authentic standard material, which would be required for positive identification, is presumed to be the penicilloic acid. Finally, a sample of pure mecillinam was degraded under highly accelerated conditions. The sample was heated at 110° for a total of 13 h. Samples taken every 3 h were assayed for mecillinam by both HPLC and UV methods. The data for these analyses are presented in Table IV. Again, good agreement is seen between the accepted procedure and the proposed HPLC assay.

TABLE IV

ACCELERATED DEGRADATION STUDY OF MECILLINAM

Exposure time at 110° (h)	*Mecillinam (%)*	
	HPLC assay	*UV assay*
0	95.7	97.2
3	96.8	98.2
6	85.7	89.5
8	77.1	79.7
13	28.2	33.5

During the course of the accelerated degradation, several impurities were observed in the HPLC chromatogram which did not correspond to any of the known compounds based on retention characteristics. In addition, the strong odor of hydrogen sulfide was noticed during heating and the sample developed an intense yellow color after a few hours. These observations suggest degradation by different mechanisms than one normally expects at under (ambient) conditions. Therefore, no attempt was made to determine accurately compounds II through VIII in these samples and, in fact, only traces of the known products were observed in the chromatograms. These observations also suggest that differences observed between the HPLC and UV analyses may be attributed to unidentified UV-absorbing impurities formed under extreme conditions.

CONCLUSIONS

The HPLC methods presented in this report are adequate to separate mecillinam from the previously identified, related compounds which may be potential impurities. In addition, mecillinam is separated from the major unidentified product of its reaction with penicillinase enzyme. The methods may be used for quantitative analysis of mecillinam and its dosage forms with acceptable accuracy and precision as compared to the published UV spectroscopic method and when two systems are employed all the compounds described can be determined quantitatively.

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