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DETERMINATION OF TAZOBACTAM AND PIPERACILLIN IN HUMAN PLASMA, SERUM, BILE AND URINE BY GRADIENT ELUTION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A gradient elution high-performance liquid chromatographic method is described for the analysis of the β-lactamase inhibitor tazobactam (YTR-830H) and a semi-synthetic parenteral penicillin, piperacillin, in human plasma, serum, bile and urine The assay for plasma, serum and bile involves deproteinization with acetonitrile and the removal of lipids with dichloromethane, urine is diluted with buffer Separation and quantitation are achieved using a mobile phase based on ion-suppression chromatography on a C_{18} reversed-phase column with ultraviolet detection at 220 nm The limit of quantitation for both compounds is 1 0 μg/ml in plasma, serum and bile using a 0 2-ml sample and 50 0 μg/ml in urine using a 0 1-ml sample The method has been validated by preparing and analyzing a series of fortified samples (range 1 0–200 μg/ml for each compound in plasma, serum and bile and 50 0–10 000 μg/ml for each compound in urine) Excellent linearity, accuracy, precision and recovery were obtained The method was not interfered with by other endogenous components, nor by other commonly administered antibiotics such as amoxicillin, mezlocillin, cefometazole and cefotaxime The assay has been successfully applied to the analysis of samples from pharmacokinetic studies in man and animals

INTRODUCTION

It was not realized until about 1960 that an important factor in determining bacterial resistance to β-lactam antibiotics might be the production of β-lactamases by bacteria [1]. These enzymes inactivate β-lactam antibiotics by opening the β-lactam ring. Research in this field also produced a considerable

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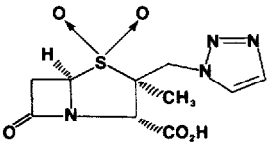
body of knowledge about these enzymes which helped in the synthesis of new cephalosporins. It was also realized that the discovery and use of β-lactamase inhibitors would extend the spectrum of enzyme-sensitive penicillins and cephalosporins

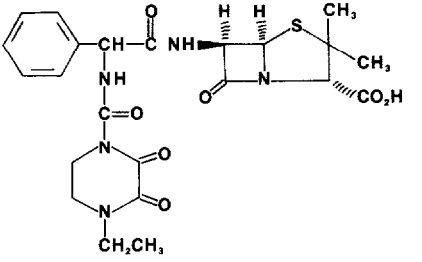
Tazobactam (YTR-830H), [2*S*-(2α,3β,5α)]-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (I), is a potent and novel β-lactamase inhibitor belonging to a class of penicillanic acid sulfones (Fig. 1). It is being co-developed as an intravenous combination with piperacillin (II), a semi-synthetic parenteral penicillin, by American Cyanamid Company (Wayne, NJ, U.S.A.) and Taiho Pharmaceutical Company (Tokushima, Japan). Compound I has been shown to act synergistically with II and a variety of other β-lactam antibiotics against a broad spectrum of bacterial pathogens [2–9].

Preliminary high-performance liquid chromatographic (HPLC) assays have been developed in-house for the determination of I and II to support pharmacokinetic and toxicokinetic studies. These assays involved isocratic techniques and tended to be time-consuming. In addition, an isocratic HPLC assay for I and a number of HPLC methods for II in biological fluids have been reported

TAZOBACTAM (I)



PIPERACILLIN (II)



BENZYLPENICILLIN POTASSIUM

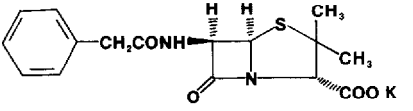


Fig 1 Structures of tazobactam (I), piperacillin (II) and benzylpenicillin potassium, the internal standard (III)

in the literature [10–16] We have recently developed a gradient elution HPLC assay to separate I and II, two compounds of widely different polarities The gradient assay has been validated for the simultaneous determination of I and II in human plasma, serum, bile and urine.

The present paper describes this HPLC method, which is not subject to interference from endogenous components, the metabolite of I or other co-administered antibiotics. The method also offers the advantage of a simultaneous assay of I and II in plasma, serum, bile and urine with reasonable simplicity, sensitivity and selectivity. Precision and accuracy of the assay, as well as stability data, are also presented.

EXPERIMENTAL

Materials and reagents

Tazobactam and piperacillin were obtained from American Cyanamid Company (Pearl River, NY, U S.A.) and Lederle-Carolina (Puerto Rico), respectively, with assigned purity greater than 95%. Monobasic sodium phosphate monohydrate, concentrated phosphoric acid and 1 *M* sodium hydroxide (all p.a. from J.T. Baker, Phillipsburg, NJ, U.S A) and acetonitrile (HPLC grade, Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used to prepare the mobile phases. The dichloromethane used in the sample preparation step was from J T Baker Benzylpenicillin potassium (III), the internal standard, was obtained from Gallard/Schlesinger (New York, NY, U.S.A.). Water was obtained daily from a Milli-Ro–Milli-Q system (Millipore, Bedford, MA, U.S.A) All chemicals and solvents were used as received without any further purification.

Standard solutions

Plasma, serum and bile. Stock solutions of tazobactam and piperacillin sodium, both 1.0 mg/ml, were prepared in methanol and deionized water, respectively. A stock solution of the internal standard (III) (5 mg/ml) was also prepared in deionized water. These solutions were found to be stable for five days at −20°C. Working stock solutions of appropriate concentrations were made by dilution of I and II in methanol and deionized water, respectively For the calibration curves, a series of blank plasma, serum and bile was spiked with each compound to give final concentrations of 1.0, 5.0, 10.0, 50.0, 100 and 200 μg/ml of I and II An appropriate aliquot of I was taken to dryness with a stream of nitrogen gas at room temperature before adding the requisite amount of II and respective biological fluid

Urine Stock solutions of I and II (both 5 mg/ml) were prepared in methanol and deionized water, respectively. A solution of the internal standard (III) (5 mg/ml) was also prepared in deionized water. A series of blank urine was spiked with each compound to give final concentrations of 50.0, 100, 200, 400,

1000, 5000 and 10 000 μg/ml for the calibration curves The methanolic aliquots of I were taken to dryness before the addition of II and urine.

Sample processing

Plasma and serum To 0 2 ml of plasma or serum, 200 μl of 0.05 *M* sodium phosphate buffer (pH 6.0) containing 25 μg/ml internal standard (III) and 800 μl of acetonitrile were added After vortex-mixing for 30 s and centrifugation at 3000 *g* for 10 min, the clear supernatant was transferred to another tube and 2 ml of dichloromethane were added. After mixing for 30 s and centrifuging at 3000 *g* for 10 min, 200 μl of the upper aqueous layer were transferred to a microinsert vial and 25 μl were injected onto the column

Bile To 0 2 ml of bile, 400 μl of 0 05 *M* sodium phosphate buffer (pH 7 0) and 2 ml of acetonitrile were added After mixing the tube for 30 s and centrifuging at 3000 *g* for 10 min, the clear supernatant was transferred to another tube and 2 ml of dichloromethane were added The tube was mixed for 30 s and centrifuged at 3000 *g* for 10 min; 200 μl of the upper aqueous phase were transferred to a microinsert vial and 25 μl were injected onto the column.

Urine To 0.1 ml of urine, 50 μl of 5 mg/ml internal standard (III) were added The vial was vortex-mixed for 30 s and the final volume was made to 10 0 ml with 0.05 *M* sodium phosphate (pH 6) buffer solution. An aliquot of the diluted urine sample was transferred to a microinsert vial and 25 μl were injected onto the column.

Instrumentation

HPLC analyses were performed using a Waters Assoc (Milford, MA, U S A) chromatograph consisting of two Model 590 solvent delivery pumps, a Model 680 gradient controller and a Model 712 WISP™ automated injector. Detection was achieved using a Kratos 783 (Applied Biosystems, Foster City, CA, U S A) variable-wavelength UV detector The detector's 0–10 mV analog signal was recorded with a Model 1200 chart recorder (Linear Instruments, Reno, NV, U S.A.) and the 0–1 V signal was digitized and recorded with a Hewlett-Packard Model 3357 laboratory data automation system (Hewlett-Packard, Paramus, NJ, U.S A). An IEC clinical centrifuge (International Equipment, Needham, MA, U S.A.) was employed to separate the plasma proteins.

HPLC conditions

All chromatographic procedures were performed at ambient temperature. Injections were made onto a Brownlee guard column (15 mm × 3.2 mm I.D., 7 μm particle size) packed with C_{18} material in series with a Keystone Hypersil ODS analytical column (250 mm × 4 6 mm I D., 5 μm particle size). The mobile phase for pump A consisted of 97% (v/v) of 0 01 *M* sodium phosphate solution (adjusted to pH 2.7 with concentrated phosphoric acid) in acetonitrile. The mobile phase for pump B consisted of 10% (v/v) of 0.01 *M* sodium phosphate

solution (pH 2.7) in acetonitrile. The mobile phases were freshly prepared and degassed by filtration. The chromatograms were generated with a linear gradient program of 95% eluent A and 5% eluent B to 50% eluent A and 50% eluent B in 9 min and a final linear step to 95% eluent A and 5% eluent B in 1 min. The total run time per sample injection on the WISP was 17 min The mobile phase flow-rate was 1.5 ml/min during the entire gradient run and the chromatogram was monitored at a chart speed of 0.25 cm/min. The UV detector was operated at 220 nm with a sensitivity of 0.02 absorbance units full scale (a u f s.). The retention times (t_R) for I, II and III were approximately 5 7, 11 8 and 12.5 min, respectively.

Data processing

Retention times for I, II and the internal standard (III) as well as the peak heights were recorded for each chromatogram with the computer system. The concentrations of I and II were determined using a calibration curve obtained by a linear least-squares regression of either the natural logarithm of the peak-height ratio of I or II to the internal standard (III) or, if an internal standard was not used, of the natural logarithm of the peak height of I or II versus the natural logarithm of the concentrations of I or II added to the biological matrices. The linearity, accuracy, precision (coefficient of variation) and recovery were determined [17]. The management of the assay sequence data with the Hewlett-Packard 3357 system has been described by Farmen et al. [18].

Assay validation

Quantitation was based on peak-height ratios for plasma, serum and urine samples and peak heights for bile samples. Standard curves were prepared by spiking the biological fluids with increasing amounts of I and II. The linearity of the assay was assessed from the limit of quantitation of each drug (i.e 1.0–200 μg/ml for plasma, serum and bile and 50.0–10 000 μg/ml for urine). Six standards were used to prepare the calibration curve for I and II in plasma, serum and bile and seven standards were used for the calibration curve in urine A 25-μl sample of each standard solution was analyzed by the present gradient HPLC method, and calibration curves were obtained by plotting the natural logarithm of the ratio of the peak height of I or II to that of the internal standard (III) against the natural logarithm of the concentration of I or II in plasma, serum or urine The calibration curves for I or II in bile were established by plotting the natural logarithm of the peak heights of I or II against the natural logarithm of the concentration of I or II. Each calibration curve for I and II was linear ($r \geqslant 0.999$).

Recovery was assessed by comparison of the absolute response (peak heights) for the processed standards to the absolute response (peak heights) for non-processed standards prepared with water rather than the matrix.

The intra-assay linearity, accuracy and precision of the method were eval-

uated in plasma, serum and bile over a concentration range of 1.0–200 μg/ml and in urine over a concentration range of 50.0–10 000 μg/ml. Triplicate samples at each concentration in the range 0 2–40 μg of either I or II were added to 0 2 ml of plasma, serum or bile and taken through the analytical procedure Similarly, triplicate samples at each concentration in the range 5–1000 μg of I or II were added to 0 1 ml of urine, diluted and injected onto the column

The inter-assay linearity, accuracy and precision of the assay were evaluated over three days. The concentrations of the solutions used were the same as those utilized for the intra-assay study.

The stability of I and II in plasma, serum and urine was assessed at room temperature (25 °C). Stability during storage at −20 °C and −70 °C was evaluated with spiked plasma. Some storage aliquots were thawed, analyzed and refrozen to assess the effect of freeze–thaw cycles on storage stability Stability of I and II in the final aqueous phase prior to injection was evaluated by repeatedly injecting a processed standard and determining the peak height or peak-height ratio as a function of time after processing.

Interference studies were carried out with some antibiotics that could be coadministered with I and II (amoxicillin, ampicillin, cefotetan, mezlocillin, cefoperazine, cefometazole, cefotaxime and cefuroxime).

As I and II were analyzed, the selectivity factor α between the two peaks was defined as $\alpha = k'_2/k'_1$ where k'_2 and k'_1 are the capacity factors of the latter- and the earlier-eluting peak, respectively The capacity factor of one peak was defined by relating the retention time (t_R) of the peak to the retention time of the unretained peak (t_0); thus, $k' = (t_R - t_0)/t_0$. Compounds I and II were completely separated from the plasma matrix with $k'_1 = 1.05$ and $k'_2 = 3\ 90$, respectively, at a flow-rate of 1.5 ml/min.

RESULTS AND DISCUSSION

A rapid, sensitive and selective gradient elution HPLC assay was developed for the simultaneous determination of I and II in human plasma, serum, bile and urine The proteins from the respective biological fluids (except urine) were precipitated with acetonitrile. Following mixing and centrifugation, the resultant supernatant was further treated with methylene chloride to remove any endogenous lipophilic materials. The sample processing and the HPLC procedures yielded chromatograms (Figs. 2–5) that were devoid of significant interference in the regions of the I, II and III peaks for plasma, serum and urine. Bile contained an endogenous component that produced a peak in the vicinity of the internal standard (III) peak As indicated from the chromatographic patterns (retention times) obtained from injections of authentic samples of other commonly administered antibiotics, none of these antibiotics had any appreciable influence on the measurement of I, II or III The retention times of amoxicillin, ampicillin, cefotetan, mezlocillin, cefoperazine, cefome-

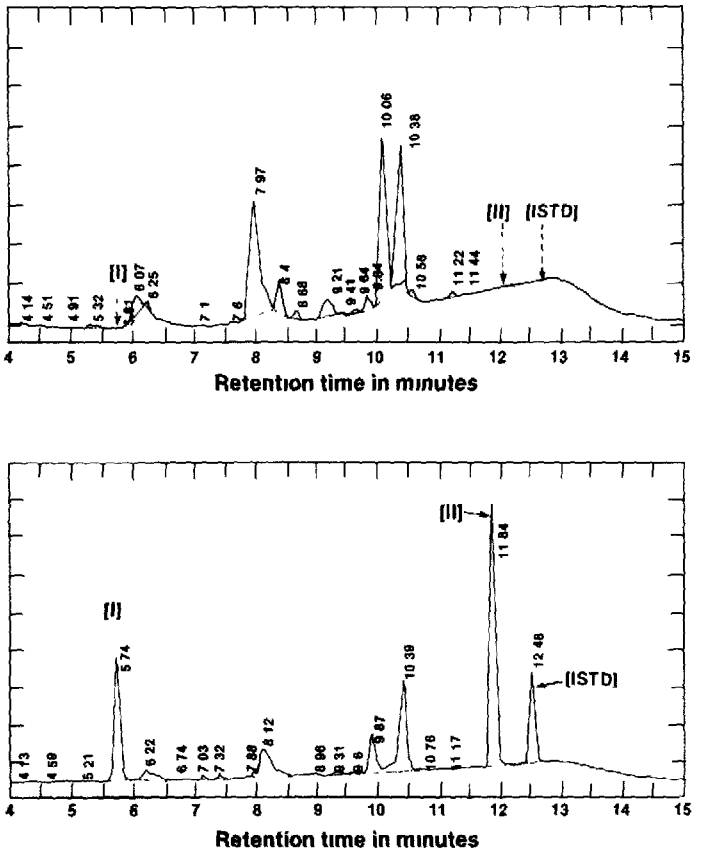


Fig 2 Chromatograms of tazobactam (I) and piperacillin sodium (II) in human plasma (Top) Control plasma, (bottom) plasma with 100 μg/ml of I and II ISTD = internal standard

tazole, cefotaxime and cefuroxime were 8.3, 11.1, 9.8, 14 2, 11.2, 10.8, 10 3 and 10.5 min, respectively. Although the specificity of the assay was demonstrated against these commonly administered antibiotics, it was not demonstrated against their possible metabolites, if any Thus, the present gradient elution HPLC assay appears to be of sufficient sensitivity and specificity for the determination of I and II in human plasma, serum, bile and urine.

Recovery, accuracy, precision and limit of quantitation

The plasma, serum, bile and urine assays were evaluated with respect to recovery, linearity of response, accuracy and precision. The UV response was a linear function ($r > 0.999$) of concentration over the ranges 1 0–200 μg/ml for plasma, serum and bile and 50 0–10 000 μg/ml for urine. The assay demonstrated excellent accuracy and precision over the concentration ranges investigated. The mean intra- and inter-assay ($n = 3$) coefficients of variation

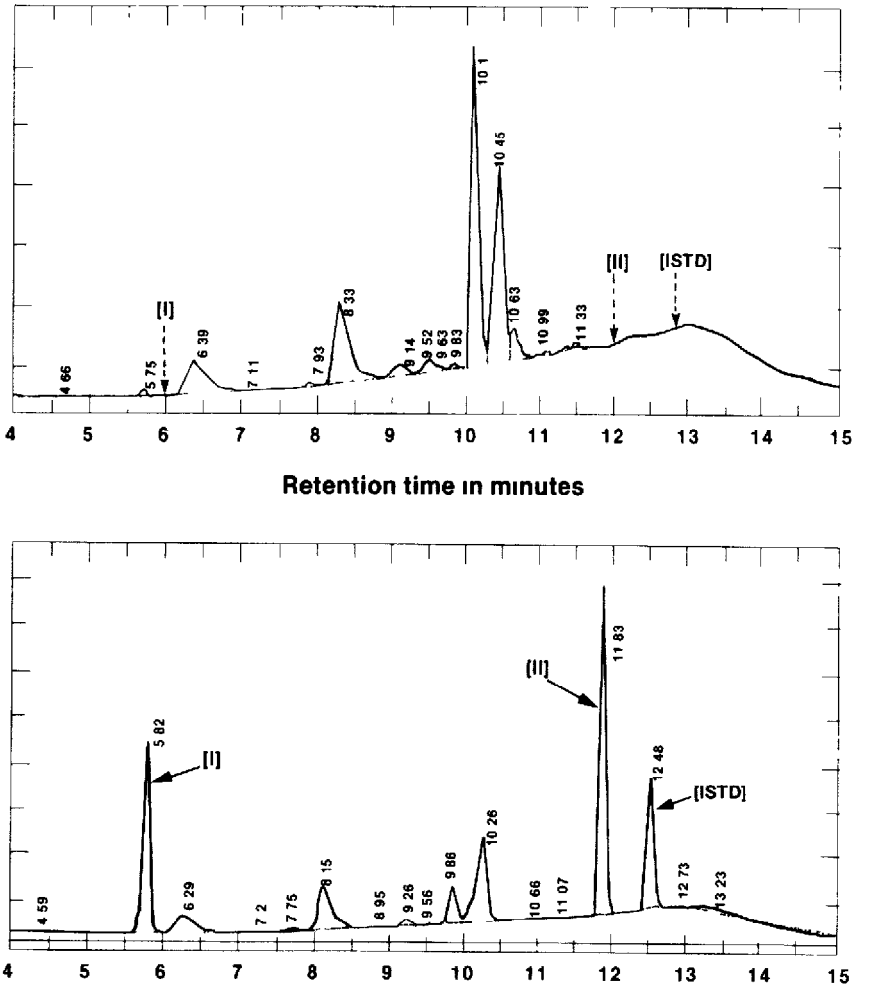


Fig 3 Chromatograms of tazobactam (I) and piperacillin sodium (II) in human serum (Top) Control serum, (bottom) serum with 100 μg/ml of I and II ISTD = internal standard

for compound I over its respective concentration ranges were 2.4 and 4.1% in plasma, 1.8 and 2.4% in serum, 3.3 and 1.8% in bile and 2.8 and 3.4% in urine, respectively. The mean intra- and inter-assay ($n=3$) coefficients of variation for compound II over its respective concentration ranges were 2.4 and 5.4% in plasma, 1.3 and 6.7% in serum, 4.6 and 6.4% in bile and 4.4 and 4.2% in urine, respectively Typical statistical validation data for compounds I and II are shown in Table I There were no statistically significant differences among the means when comparing the intra- and inter-day precision data ($P > 0.05$, Student's t-test).

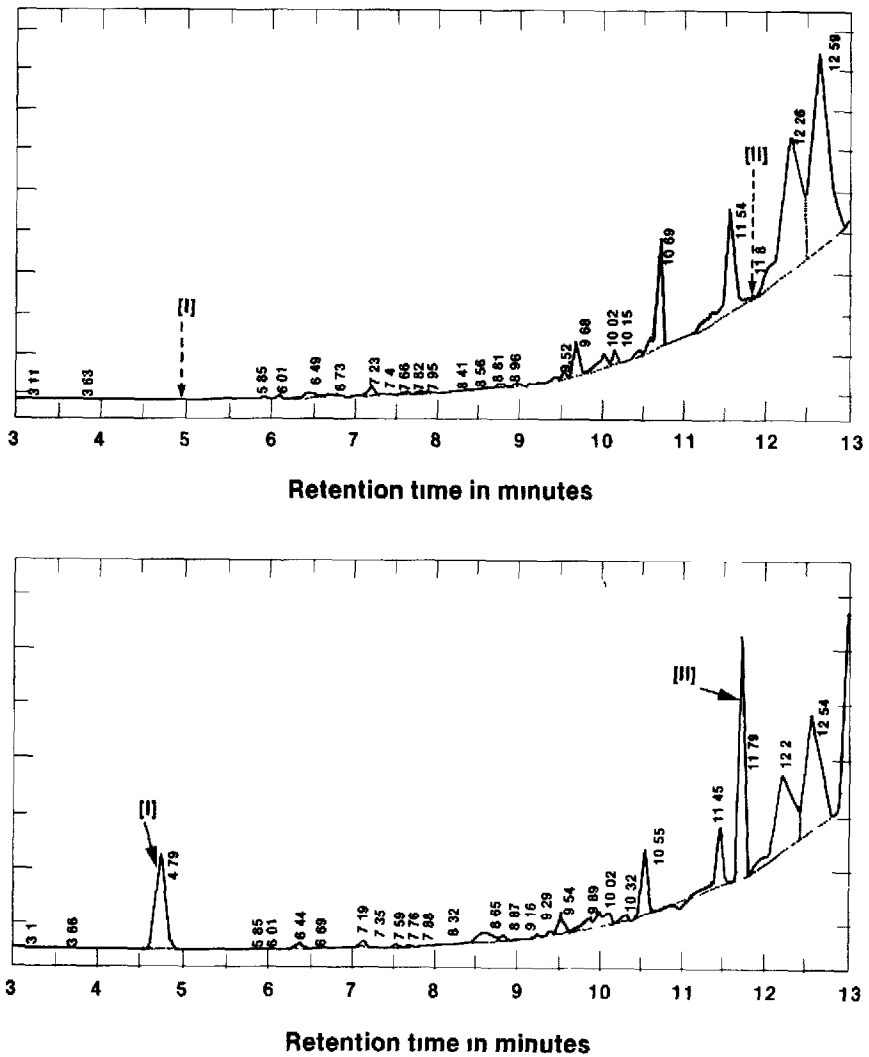


Fig 4 Chromatograms of tazobactam (I) and piperacillin sodium (II) in human bile (Top) Control bile, (bottom) bile with 100 μg/ml of I and II

The mean ($\pm$S.D) recovery of compounds I and II in the biological fluids evaluated (based on absolute response) was 98.8 $\pm$ 4.5 and 104 $\pm$ 7.1%, respectively The limit of quantitation was 1.0 μg of I or II per ml of plasma, serum and bile and 50.0 μg of I or II per ml of urine.

Stability

Compounds I and II were found to be stable in solution at room temperature for up to 20 h following the extraction procedure described in this report; hence

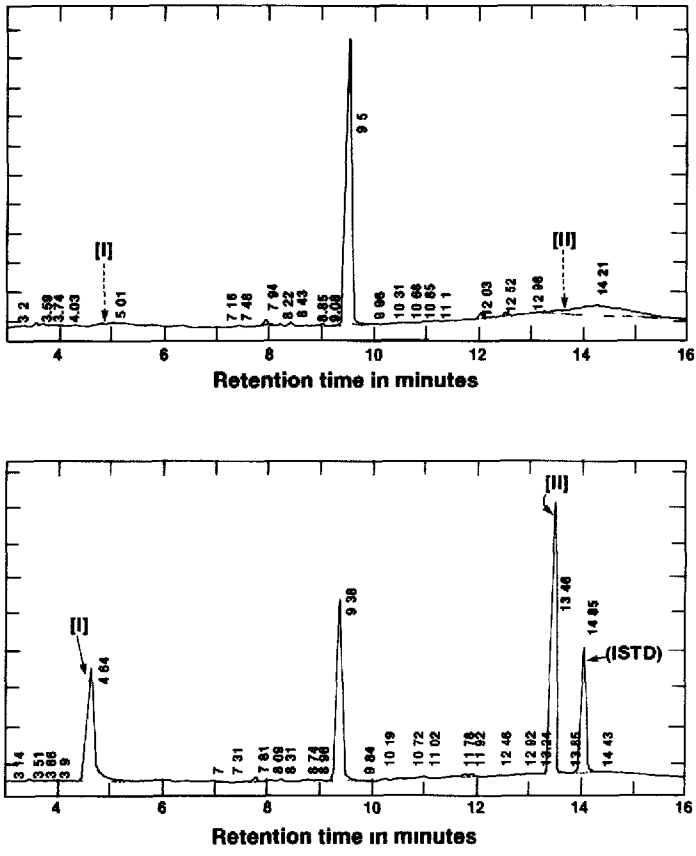


Fig 5 Chromatograms of tazobactam (I) and piperacillin sodium (II) in human urine (Top) Control urine, (bottom) urine with 5000 μg/ml of I and II ISTD = internal standard

the present assay can be used with an automated injector for automated runs. At least three freeze–thaw cycles can be tolerated without substantial (more than 10%) loss of activity of either I or II in plasma and urine. The stability of I and II in biological fluids was also assessed at room temperature, −20 °C and −70 °C. Compounds I and II were stable up to a period of 1 h at room temperature in plasma and serum. Compound I exhibited a loss of 11–15% in plasma and serum after 4 h while compound II showed a loss of 21–29% under the same time period. Compound I was stable in bile up to a period of 4 h and indicated a loss of 11% after 24 h. Compound II was stable up to 1 h in bile and showed a degradation of 20 and 75% after 4 and 24 h, respectively. Both compounds were stable in human urine up to 24 h. Both compounds showed no discernible degradation over a nine-day period at −20 °C in human plasma, but were unstable thereafter. Compound I was stable up to 180 days at −70 °C

TABLE I

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR TAZOBACTAM (I) AND PIPERACILLIN (II) IN HUMAN PLASMA

Added concentration (μg/ml)	Found concentration (mean ± S D) (μg/ml)	Coefficient of variation (%)
I intra-day (n=3)[a]		
1 0	0 90 ± 0 03	3 3
5 0	5 49 ± 0 08	1 5
10 0	10 8 ± 0 53	4 9
50 0	50 2 ± 0 76	1 5
100	97 2 ± 0 80	0 8
200	193 ± 5 10	2 6
		Mean 2 4
I inter-day (n=3)[b]		
1 0	0 96 ± 0 02	2 1
5 0	5 69 ± 0 37	6 5
10 0	10 4 ± 0 50	4 8
50 0	48 3 ± 2 14	4 4
100	95 3 ± 2 35	2 5
200	203 ± 9 23	4 5
		Mean 4 1
II intra-day (n=3)[a]		
1 0	1 01 ± 0 04	4 0
5 0	5 04 ± 0 08	1 6
10 0	9 50 ± 0 54	5 7
50 0	52 5 ± 0 20	0 4
100	98 6 ± 1 49	1 5
200	200 ± 2 90	1 4
		Mean 2 4
II inter-day (n=3)[b]		
1 0	0 98 ± 0 05	5 1
5 0	5 31 ± 0 41	7 7
10 0	9 62 ± 0 17	1 8
50 0	49 4 ± 4 33	8 8
100	95 9 ± 2 66	2 8
200	208 ± 13 1	6 3
		Mean 5 4

[a]Three separately spiked samples at each of six concentrations
[b]On three different days, plasma spiked at six concentrations and analyzed

in human plasma while compound II was only stable up to 75 days under the same storage conditions

Application of the assay to biological samples

This assay has been successfully applied to the analysis of samples from pharmacokinetic studies in man and animals. Routinely, 40–50 injections have been made in each assay sequence with predictable results and no indication of chromatographic interferences. When applied in other laboratories, changes in sample handling and instrumentation were almost unavoidable Therefore, quality control samples were prepared, stored, thawed and analyzed with each set of study samples. Verification of analysis validity was based on the precision and accuracy of the quality control results as well as the standard curve statistics.

CONCLUSION

The gradient elution HPLC assay described in this report is suitable for monitoring I and II in pharmacokinetic studies. The assay is sensitive, specific and reproducible to allow precise measurements of I and II in human plasma, serum, bile and urine

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