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Determination of piperacillin and mezlocillin in human serum and urine by high-performance liquid chromatography after derivatisation with 1,2,4-triazole

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Abstract

High-performance liquid chromatographic methods have been developed for the determination of piperacillin and mezlocillin in human serum and urine samples. The methods involve ultrafiltration of samples followed by reaction with 1.5 *M* 1,2,4-triazole and $0.5 \cdot 10^{-3}$ *M* mercury(II) chloride in solution (pH 8.50) at 50°C for 15 min. The resulting products were separated on a C_{18} column following stabilisation in an eluent containing sodium thiosulphate. They were detected at 323 nm for both penicillins. The methods have been applied to assays of these penicillins in human serum and urine samples. The procedures, which permit the determination of penicillin concentration down to 0.1 $\mu g\,ml^{-1}$ in serum and 1 $\mu g\,ml^{-1}$ in urine samples, are specific to intact penicillins without interference from corresponding penicilloates [see J. Haginaka et al., Anal. Sci. 1 (1985) 73]. At concentrations of 1–500 $\mu g\,ml^{-1}$ for each compound, the within- and between-day precisions were 1.8–4.8 and 3.7–6.9, respectively. The accuracy was ca. 100% for all samples assayed.

Keywords: Derivatisation, LC; Piperacillin; Mezlocillin; Triazole; Penicillins; Antibiotics

1. Introduction

A number of papers on HPLC of piperacillin [1–8] and mezlocillin [2,9–13] describe the determination of these antibiotics in biological samples. The reversed-phase mode is most commonly used, with precipitation, ultrafiltration and extraction of serum proteins prior to injection. Ultraviolet (UV) detection methods at around 220 nm, which lack both sensitivity and selectivity have been mainly used for the assay of these penicillins. In order to enhance detectability, precolumn derivatisation procedures have been investigated [14–20].

In this paper, we describe the use of 1,2,4-triazole mercury(II) chloride in a precolumn derivatisation procedure for the HPLC determination of piperacillin and mezlocillin in serum and urine samples.

2. Experimental

2.1. Reagents and materials

Mezlocillin and piperacillin were purchased from Sigma (St. Louis, MO, USA). 1,2,4-Triazole, acetonitrile and other chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, Germany).

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1,2,4-Triazole reagent solution 1.5 *M* pH 8.50 containing $0.5 \cdot 10^{-3}$ *M* mercury(II) chloride was prepared as follows: 10.36 g of 1,2,4-triazole was dissolved in 70 ml of distilled water; a 10-ml volume solution of mercury(II) chloride (0.135 g dissolved in water and diluted to 100 ml) was then added and the pH adjusted to 8.50 by the addition of saturated sodium hydroxide solution. This was then diluted to 100 ml.

The standard solutions used to spike the serum and urine samples were prepared by dissolving 0.001 g of the appropriate penicillin in 1 ml of distilled water.

The human serum and urine controls were provided by the clinical analysis service of the León Hospital Complex.

All water used in this study was purified with a Milli-Q water purification system (18 MΩ resistance) (Millipore, Bedford, MA, USA).

Ultrafiltration tubes (Ultrafree C3LGC, low binding cellulose, molecular mass cut-off 10 000) were from Nihon Millipore (Yonezawa, Japan).

2.2. Instruments and chromatography

Precolumn reaction conditions were examined and penicillins assayed with a HPLC System Gold liquid chromatograph from Beckman Instruments (Fullerton, CA, USA) equipped with a programmable solvent Module 116 pump and a Module 166 variable-wavelength UV–visible detector and a Module 168 photodiode array detector system. Samples were loaded onto the column via a Rheodyne 7125 loop injector (volume 50 μl) and a 150×4.6-mm I.D. Nucleosil C_{18} column (5 μm particle size) (Macherey-Nagel, Düren, Germany) was used, protected with a precolumn (30×4.6 mm I.D.) packed with the same material.

2.3. Chromatographic conditions

The mobile phase used was as follows: 0.1 *M*, pH 6.00, sodium phosphate–0.02 *M* sodium thiosulphate–acetonitrile solution (1:1:1, v/v). The flow-rate was maintained at 1 ml min^{-1}. All separations were carried out at ambient temperature and detection of reaction product performed at 323 nm. The reaction products of piperacillin and mezlocillin with 1,2,4-triazole and mercury(II) chloride were stabilised in a mobile phase containing sodium thiosulphate. As revealed by Rogers et al. [16,17], the absence of this reagents resulted in very broad, asymmetrical peaks.

2.4. Pre-column derivatisation procedure

2.4.1. Serum samples

A penicillin standard was dissolved in a human serum control sample. A 400-μl aliquot of the serum sample was ultrafiltered using an Millipore ultrafree C3LGC at 5000*g* at ambient temperature. To a 100-μl aliquot of the ultrafiltrate, 200 μl of 1.5 *M* 1,2,4-triazole reagent (pH 8.5) was added. The solution was sealed in a screw-top vial and allowed to stand in a water-bath at 50°C for 15 min. The vial was removed and immediately cooled to room temperature. A 50-μl portion of the mixture was loaded onto the HPLC column.

2.4.2. Urine samples

A penicillin standard was dissolved in a urine sample, which diluted ten-fold with water, was filtered with a 0.45-μm filter (Millex-HA, Millipore). The filtrate was treated and analysed according to the same procedures as those for serum samples. A 50-μl portion of the mixture was loaded onto the HPLC column.

2.5. Quantification

The serum and urine calibration standard solutions of the antibiotics were prepared at ten different concentrations between 0.4 and 100 μg ml^{-1} for serum and between 4 and 500 μg ml^{-1} for urine, and treated in the manner described above. Calibration graphs of the chromatographic peak-areas versus antibiotic concentration were constructed. Antibiotic concentrations in the unknown serum and urine samples were calculated by interpolation from the calibration graphs by a least-squares regression line treatment.

3. Results and discussion

3.1. UV absorption spectra

The ultraviolet spectrum between 250 and 500 nm of the reaction product of each penicillin with 1,2,4-triazole reagent solution run against the 1,2,4-triazole reagent solution as a reference shows a maximum of about 323 nm (Fig. 1). This product proved to be stable for 30–35 min at room temperature, the stability increased until 45–50 min working at 4°C. The molar absorptivity was about $1.69 \cdot 10^4$ $l\,mol^{-1}\,cm^{-1}$ for mezlocillin and $1.09 \cdot 10^4$ $l\,mol^{-1}\,cm^{-1}$ for piperacillin.

3.2. Reaction conditions for piperacillin

The factors affecting the reaction of the penicillins with 1,2,4-triazole reagent, such as pH, concentration of the reagent, concentration of mercuric chloride and reaction temperature were examined in aqueous solutions by plotting the peak areas at 323 nm against the reaction time.

(1) Effect of pH: Fig. 2 shows the effect of pH (8.00, 8.50, and 9.00) on the formation of the derived product for the reaction of piperacillin with 1 *M* (final concentration) of 1,2,4-triazole reagent containing $0.5 \cdot 10^{-3}$ *M* mercuric chloride at 50°C. As one can see, when the pH of the reaction solution was high (pH 9.00), the reaction was faster but the reaction product was less stable, so the maximum peak area after a reaction time of 10 min was two thirds that obtained after 5 min.

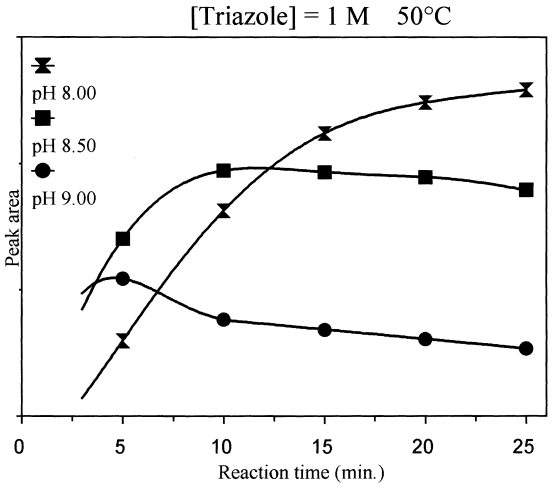

Fig. 2. Effects of pH using 1 *M* 1,2,4-triazole reagent at 50°C on the precolumn derivatisation of piperacillin.

(2) Effect of 1,2,4-triazole concentration: The effect of triazole concentration was not as great as that of pH. Fig. 3 shows the effect of the charge in

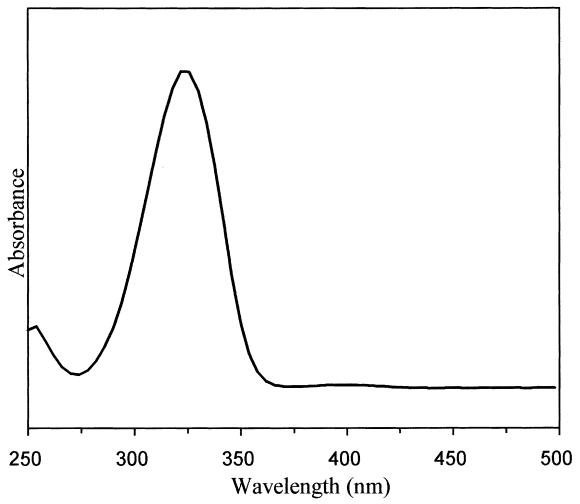

Fig. 1. Ultraviolet spectrum of the reaction products of piperacillin with 1,2,4-triazole.

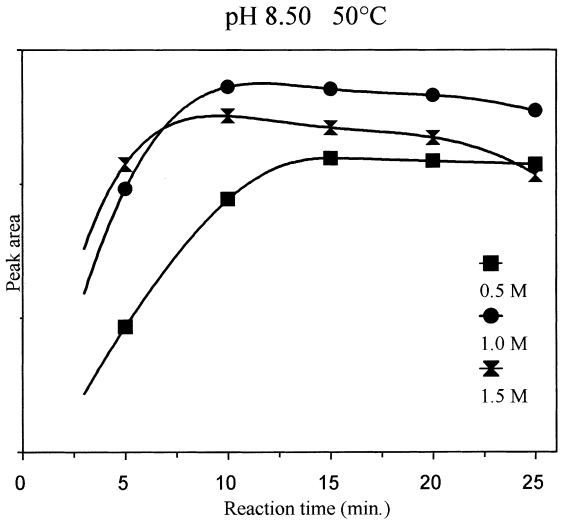

Fig. 3. Effects of 1,2,4-triazole concentration using 1,2,4-triazole reagent pH 8.50 at 50°C on the precolumn derivatisation of piperacillin.

the 1,2,4-triazole final concentration between 0.5 *M* and 1.5 *M* at pH 8.50, 50°C and a mercuric chloride concentration of $0.5 \cdot 10^{-3}$ *M*. These results show that the reaction with 1,2,4-triazole was faster at a concentration of 1.5 *M* than at 0.5 *M* or 1 *M*, but at 1.5 *M*, the peak areas are smaller than at 1 *M*.

(3) Effect of temperature: The effects of varying the reaction temperature (40°C, 50°C, 60°C) on the formation of a mercaptide at pH 8.50, 1 *M* 1,2,4-triazole reagent is significant. Fig. 4 shows that peak areas were lower after reaction at 40 and 60°C than at 50°C. At high temperature the chromophore forms more quickly, but it degrades and a slight drop in peak areas is noticed.

(4) Effect of mercuric chloride concentration: No appreciable effect of mercuric chloride concentration of the reaction rate and maximum peak areas were observed over the range $0.1 \cdot 10^{-3}$ to $1 \cdot 10^{-3}$ *M*. Thus, the concentration of mercuric chloride was fixed at $0.5 \cdot 10^{-3}$ *M*.

The penicillins studied, apart from reacting to give the corresponding mercuric mercaptide, are degraded in processes of hydrolysis, especially at high pH values and high temperatures. This degradation of the intact penicillin and mercaptide formed must be the reason for the differences in the peak area reached under different conditions. For example, at pH 9.00, these penicillins are partially hydrolysed before the formation of the mercaptide, which in turn is very unstable and is partially degraded as it is formed. Because of this, the conditions selected for piperacillin assay were 15 min at 50°C in 1 *M* (final concentration) of 1,2,4-triazole and $0.5 \cdot 10^{-3}$ *M* mercury(II) chloride solution at pH 8.50. After the 15-min reaction time, the samples were cooled to 20–25°C and injected onto the HPLC system.

3.3. Reaction conditions for mezlocillin

The assay conditions selected for mezlocillin were the same as for piperacillin. Fig. 5 shows the time courses of the formation of UV-absorbing products from the penicillins studied in the assay conditions. It may be observed that the chromophores formed under these conditions are stable.

3.4. Reaction products

We have not identified the products of the reactions between 1,2,4-triazole and the penicillins studied, but it is obviously a mercuric mercaptide as it does not form in the absence of Hg^{2+}. Haginaka et al. [1] reported that the reaction of other penicillins with 1,2,4-triazole in the presence of mercuric chloride might yield the corresponding penicillenic acid–mercury mercaptide (III) via the intermediate

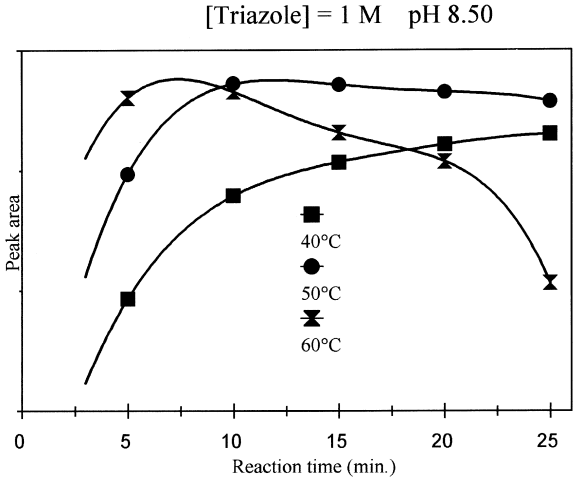

Fig. 4. Effects of temperature using 1,2,4-triazole reagent at pH 8.50 on the precolumn derivatisation of piperacillin.

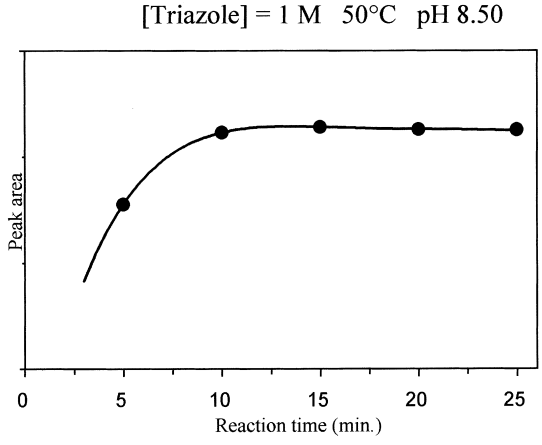

Fig. 5. Effects of reaction time on the precolumn derivatisation of mezlocillin.

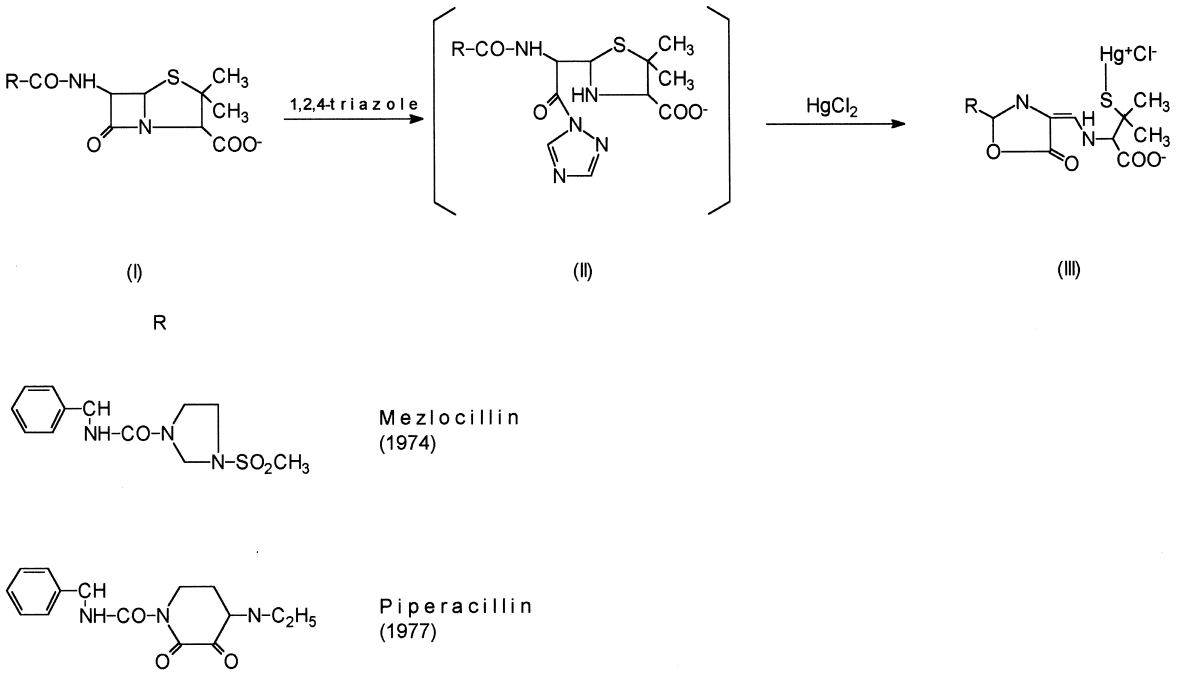

Fig. 6. Possible reactions of mezlocillin and piperacillin with the 1,2,4-triazole reagent.

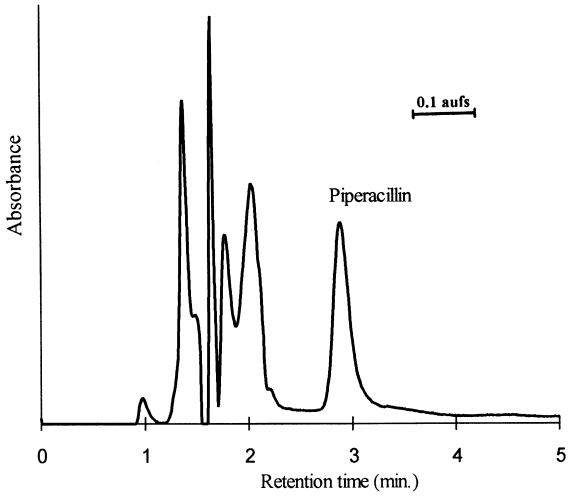

Fig. 7. Chromatogram of piperacillin chromophore from human serum spiked with piperacillin at 20 μg ml^{-1}. See Section 2 for chromatographic conditions.

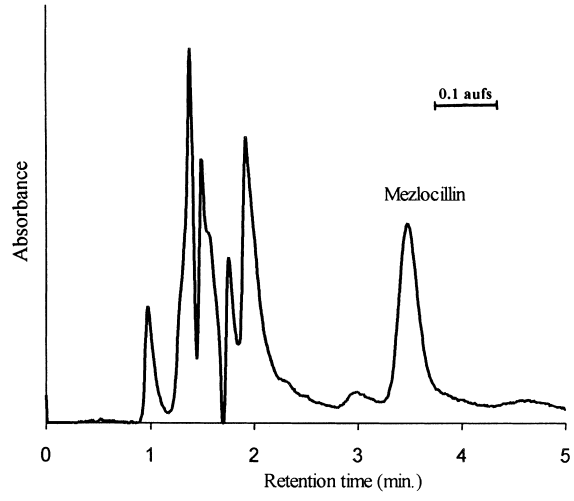

Fig. 8. Chromatogram of mezlocillin chromophore from human serum spiked with mezlocillin at 20 μg ml^{-1}. See Section 2 for chromatographic conditions.

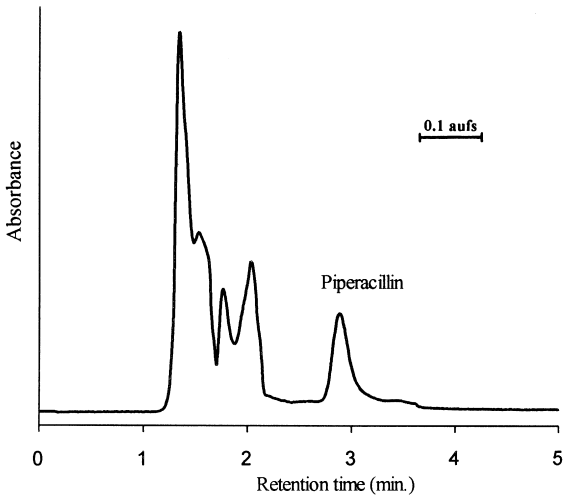

Fig. 9. Chromatogram of piperacillin chromophore from human urine spiked with piperacillin at 100 μg/ml. See Section 2 for chromatographic conditions.

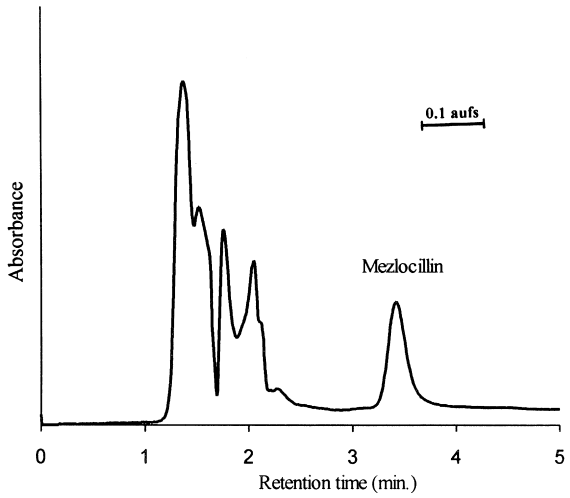

Fig. 10. Chromatogram of mezlocillin chromophore from human serum spiked with mezlocillin at 100 μg/ml. See Section 2 for chromatographic conditions.

of *N*-penicilloyl-1,2,4-triazole (II) (Fig. 6). The reactions of piperacillin and mezlocillin with the 1,2,4-triazole reagent might also yield the corresponding penicillenic acid–mercury mercaptide (III).

3.5. Selectivity

Figs. 7–10 show the chromatograms from analysis of piperacillin and mezlocillin in human serum and urine samples. Under the conditions used, piperacillin and mezlocillin were well resolved from endogenous serum or urine compounds. Minor changes to the organic modifier content of the mobile phase were occasionally required to accommodate column efficiency loss, or interference from atypical serum and urine samples.

3.6. Recovery

The total recoveries of the penicillins were measured on blank serum and urine spiked with these antibiotics at different concentrations. The detector responses to spiked samples were compared with those to 50 m*M* phosphate buffer solutions (pH 6.00) with identical concentrations of the antibiotics under study. The results showed that the main drawbacks are that ca. 5% of the antibiotics are absorbed in the ultrafiltration membrane. Therefore the ultrafiltration method for removing proteins from serum has a good level of efficacy. Table 1 shows the total recoveries of piperacillin and mezlocillin from spiked serum and urine samples.

3.7. Linearity and sensitivity

For both penicillins we found a good linear relationship between the peak-area ratios and the penicillin concentrations in serum and urine samples in the range studied, with regression analysis of the data revealing a correlation coefficient of ≥ 0.998 for both penicillins. We estimated that the limits of determination were 0.1 $\mu g\,ml^{-1}$ for piperacillin and 0.2 $\mu g\,ml^{-1}$ for mezlocillin, with a signal-to-noise ratio of approximately 3.

Table 1
Recoveries of piperacillin and mezlocillin from human serum and urine samples

Sample	Concentration added ($\mu g\,ml^{-1}$)	Recovery (mean±S.D.) (%) ($n=6$)	
		Piperacillin	Mezlocillin
Serum	5	98.2±3.3	97.3±1.7
	25	96.9±3.6	101±4.8
Urine	10	102±5.1	98.9±4.1
	100	95.3±4.8	103±3.4

Table 2
Accuracy and precision results for human serum and urine samples spiked with piperacillin

Concentration (mean±S.D.) (μg ml^{-1})		Accuracy[a] (%)	Precision[b] (%)
Added	Found		
Serum			
Within-day (n=6)			
1	0.97±0.04	97	4.1
50	48.3±1.81	97	3.7
Between-day (n=6)			
5	5.18±0.22	104	4.2
100	102±4.72	102	4.6
Urine			
Within-day (n=6)			
5	5.10±0.09	102	1.8
250	245±7.15	98	2.9
Between-day (n=6)			
10	9.87±0.51	99	5.2
500	510±35.3	102	6.9

[a] (Found/added)×100.
[b] R.S.D.

Table 3
Accuracy and precision results for human serum and urine samples spiked with mezlocillin

Concentration (mean±S.D.) (μg ml^{-1})		Accuracy[a] (%)	Precision[b] (%)
Added	Found		
Serum			
Within-day (n=6)			
1	1.02±0.03	102	2.9
50	47.9±1.03	96	2.1
Between-day (n=6)			
5	4.98±0.23	100	4.6
100	101±3.71	101	3.7
Urine			
Within-day (n=6)			
5	4.96±0.18	99	3.6
250	255±12.1	102	4.8
Between-day (n=6)			
10	9.62±0.37	96	3.8
500	489±25.6	98	5.2

[a] (Found/added)×100.
[b] R.S.D.

3.8. Accuracy and precision

Known amounts of piperacillin and mezlocillin were added to control serum and urine to give three samples in the concentration range 1–100 μg ml^{-1}. Sufficient serum was prepared to allow six replicate samples to be analysed at each concentration. The procedure was repeated for urine over the concentration range 5–500 μg ml^{-1}. The results are shown in Tables 2 and 3 from which one can see that the relative standard deviations (R.S.D.s) ranged from 1.76 to 6.92% for within-day and between-day reproducibility. The accuracy, defined as (amount found/amount added)×100 (%) was ca. 100% for all samples assayed.

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