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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMPICILLIN AND ITS METABOLITES IN RAT PLASMA, BILE AND URINE BY POST-COLUMN DEGRADATION WITH SODIUM HYPOCHLORITE

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of ampicillin (1) and its metabolites [(5*R*,6*R*)-ampicilloic acid (2), the (5*S*,6*R*)-epimer (3) and the corresponding (2*R*)-piperazine-2',5'-dione (4)] in rat plasma, bile and urine. The method involves the separation of 1–4 from the background components of the biological fluids on a reversed-phase C_{18} column, using sodium heptanesulphonate as an ion-pairing agent and methanol in the mobile phase, followed by post-column degradation with 1.5 *M* sodium hydroxide–0.02% sodium hypochlorite solution at ambient temperature, and detection of the degradation product(s) of each compound at 270 nm. The detection limits were about 25 ng for each compound at a signal-to-noise ratio of 3. At concentrations of 2–5 μg/ml of each compound, the within- and between-run precisions (relative standard deviation) were 0.77–7.15% and 1.76–5.96%, respectively. The plasma, biliary and urinary levels of 1 and its metabolites were determined by the proposed method after intravenous administration of 1 to rats.

INTRODUCTION

The assay of ampicillin (1) in pharmaceutical preparations and in biological samples by high-performance liquid chromatography (HPLC) has been based mainly on direct UV detection at around 220 nm[1–13]. Owing to their selectivity and sensitivity, derivatization techniques in HPLC can be advantageous. For the assay of 1 in biological fluids, several HPLC methods, combined with pre-column[14,15] and post-column[16–20] derivatization, have been developed.

It is well known that penicillins are metabolized to the corresponding (5*R*,6*R*)-penicilloic acids and the (5*S*,6*R*)-epimers in man[21,22]. In previous papers[23,24], we reported that ampicillin piperazine-2,5-dione (4) is excreted as a metabolite in human urine, and we described an HPLC method for the determination of 1 and its metabolites [(5*R*,6*R*)-ampicilloic acid (2), the (5*S*,6*R*)-epimer (3) and 4]

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in human urine by post-column alkaline degradation with sodium hydroxide, mercury(II) chloride and ethylenediaminetetraacetic acid in solution. However, those methods have the disadvantage that they require the use of mercury(II) chloride (which is toxic to humans and an environmental pollutant) at a high concentration. Previously, we reported HPLC methods for the determination of penicillins[20] and amoxicillin and its metabolites in human urine[25], including post-column reaction with sodium hypochlorite. This paper deals with the application of this method to the simultaneous determination of 1 and its metabolites (2, 3 and 4) and pharmacokinetic studies following the intravenous administration of 1 to rats.

EXPERIMENTAL

Materials

The structures of 1 and its metabolites (2,3 and 4) are illustrated in Fig. 1. The sodium salt of 1 was kindly donated by Toyo Jozo (Shizuoka, Japan), 2 and 3 were prepared according to the method reported previously[22] and 4 was obtained as described previously.[6]

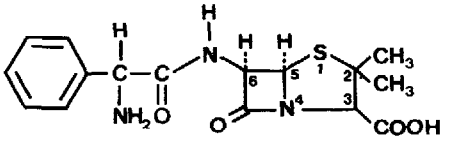
Sodium heptanesulphonate, sodium hypochlorite solution (Antiformin), and other chemicals of analytical-reagent grade were obtained from Nakarai Chemicals (Kyoto, Japan) and used without further purification. Deionized, glass-distilled water and distilled methanol were used to prepare the HPLC eluents.

Chromatography

The experimental setup used in this study included a Trirotar-V pump (Japan Spectroscopic, Tokyo, Japan) and an NP-DX-2 pump (Nihon Seimitsu Kagaku, Tokyo, Japan) for delivering the eluent and post-column reagent, respectively; a Model VL-614 loop injector (Japan Spectroscopic), equipped with a 200-μl loop; a 150 × 4.6 mm I.D. Nucleosil-5 C_{18} column (5-μm particle size) (Macherey, Nagel & Co., Düren, F.R.G.), protected with a pre-column (30 × 4.6 mm I.D.), packed with the same material; a Diflon tee-piece (each angle 120°); an open-tubular post-column reactor (PTFE tubing, 1 m × 0.5 mm I.D.); a Uvidec-100-V spectrophotometer (Japan Spectroscopic), equipped with an 8-μl flow-through cell; and a C-R3A recorder/integrator (Shimadzu, Kyoto, Japan). The mobile phases used were as follows: eluent A, 10 m*M* NaH_2PO_4–10 m*M* Na_2HPO_4–methanol (1:1:1, v/v); eluent B, 15 m*M* sodium heptanesulphonate–3 m*M* NaH_2PO_4–27 m*M* H_3PO_4–methanol (1.6:1.6:1.6:3, v/v); and eluent C, 10 m*M* sodium heptanesulphonate–100 m*M* H_3PO_4–methanol (0.8:0.8:1, v/v). Eluents A, B and C were used for the assays of 1 and its metabolites in plasma, bile and urine, respectively. The flow-rate was maintained at 0.8 ml/min. The post-column reagent used was 1.5 *M* sodium hydroxide–0.02% sodium hypochlorite solution, the flow-rate of which was maintained at 0.2 ml/min. Detection was performed at 270 nm. All separations and post-column reactions were carried out at ambient temperature.

Pre-treatment

For plasma samples, 100 μl of 10 *M* urea solution were added to a 100-μl aliquot of the plasma sample and the mixture was ultrafiltered using an Amicon MPS-1 micropartition system (Amicon, Tokyo, Japan) with an Amicon YMT mem-



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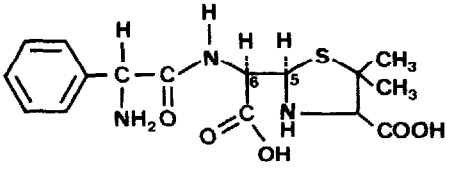

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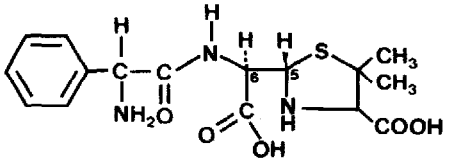

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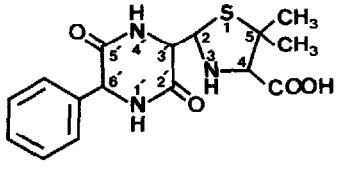

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Fig. 1. Structures of ampicillin and its metabolites: 1, ampicillin; 2, (5*R*,6*R*)-ampicilloic acid; 3, (5*S*,6*R*)-ampicilloic acid; 4, (2*R*)-piperazine-2′,5′-dione.

brane at 1500 *g* for 10 min at ambient temperature. A 20–50-μl portion of the ultrafiltrate was loaded on to the column.

For bile samples, the bile sample, diluted 5–10-fold with eluent B, was centrifuged at 8000 *g* for 5 min at ambient temperature. A 20–50-μl portion of the supernatant was loaded on to the column.

For urine samples, the urine sample, diluted 5–20-fold with water, was filtered through a 0.45-μm acrylate copolymer membrane (Gelman Science Japan, Tokyo, Japan). A 20–50-μl portion of the filtrate was loaded on to the column.

Rat experiments

Three male Wistar rats (235–255 g) were used. Under pentobarbitone anaesthesia, the sodium salt of 1 (100 mg/kg), dissolved in 0.9% sodium chloride solution, was rapidly injected into the jugular vein. A blood sample (0.25 ml) was collected from the jugular vein at 0, 20, 40, 60, 120 and 240 min with a heparinized syringe. The plasma layer was obtained by centrifugation of the blood at 1500 *g* for 10 min, and stored at −20°C until taken for assay. The bile and urine samples were transferred through a polyethylene tube inserted into the bile duct or ureter to a test-tube, chilled with crushed ice, and stored at −20°C until assayed. A 1-ml volume of water was previously added to the test-tube for the collection of the urine sample.

Calibration graph

Standard solutions were prepared by dissolving known amounts of 1, 2, 3 and 4 in control rat plasma, bile and urine to make five to seven different concentrations and were treated according to the procedures described above. The concentration ranges were as follows: 1, 10–1000 μg/ml; 2 and 3, 2–50 μg/ml; and 4, 1–20 μg/ml. The calibration graph was constructed as peak area (1) or peak height (2, 3 and 4) *versus* concentration.

Pharmacokinetics

The area under the plasma concentration–time curve (AUC), the mean residence time (MRT), the steady-state distribution volume (Vss), the total body clearance (CL_T) and the percentage of the dose excreted in bile and urine up to time infinity (f_{bil} and f_e) were calculated by the method of Yamaoka *et al.*[26,27].

RESULTS AND DISCUSSION

Post-column reaction conditions

Previously, we reported[20] that the reaction of penicillins with sodium hypochlorite proceeded faster in the presence of methanol than in its absence to yield products with UV absorption maxima at *ca.* 280 and 270 nm, respectively, and that the former reaction gave a higher absorbance than the latter. The reaction in the presence of methanol is preferable to that in its absence for the present purpose. Therefore, the various factors affecting the post-column reaction were examined by flow injection analysis in the presence of methanol. The carrier solution (eluent B) and post-column reagent were delivered at flow-rates of 0.8 and 0.2 ml/min, respectively, and the reaction-coil length (0.5 mm I.D.) was fixed at 1 m (corresponding to a residence time of 12 s). Fig. 2 shows the effect of the concentration of sodium hydroxide on the UV response at a sodium hypochlorite concentration of 0.02%. The maximal UV response was obtained at a sodium hydroxide concentration of 0.5 *M* for 1, whereas for 2, 3 and 4 the optimal concentration of sodium hydroxide was 1.5 *M*. The concentration of sodium hypochlorite was changed from 0.005 to 0.04% at a sodium hydroxide concentration of 1.5 *M* to give the maximal UV response at 0.02% for all compounds. Thus, the post-column reaction conditions described under Experimental were selected for the simultaneous determination of 1, 2, 3 and 4. The detection wavelength was examined by changing the wavelength of the HPLC detector. The optimal detection wavelengths were 280 nm for 1, 258 nm for 2, 262 nm for

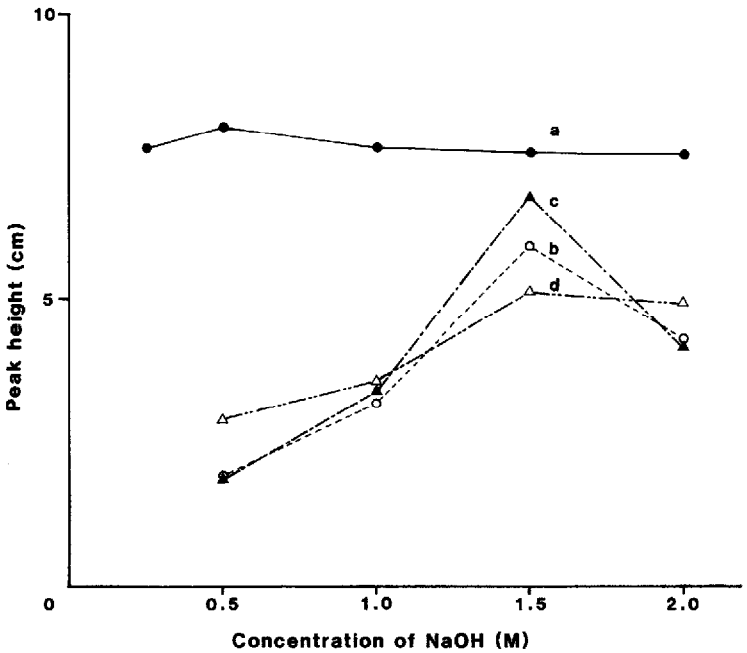

Fig. 2. Effect of sodium hydroxide concentration on the UV absorbance of the degradation products of (a) 1, (b) 2, (c) 3 and (d) 4, determined by flow-injection analysis. Other parameters were kept constant: sodium hypochlorite concentration, 0.02%; coil length, 1 m. A 20-μl portion of each sample solution (25 μg/ml) was injected at a detector sensitivity of 0.128 a.u.f.s. for 1 and 0.064 a.u.f.s. for the other compounds.

3 and 252 nm for 4. However, with detection at lower wavelengths the background components of bile and urine interfered in the assay of 2, 3 and/or 4. Therefore, detection was performed at 270 nm, where 1–4 were about 50–90% as sensitive as at the optimal wavelength.

Pre-treatment

When the plasma samples were ultrafiltered without pre-treatment or after dilution with an equal volume of borate buffer solution, the recoveries of 1 were 81 and 75%, respectively. However, ultrafiltration followed by denaturation of the plasma proteins with urea gave a total recovery above 97% (as shown in Table I). Therefore, the pre-treatment procedures described under Experimental were adopted for plasma samples. Direct injection of bile and urine samples resulted in the disappearance of the peaks of 2 and 3, or in deteriorated peak shapes. Therefore, the bile and urine samples were diluted 5–20-fold.

HPLC separation and detection of 1 and its metabolites

Previously reported HPLC methods, combined with pre- and post-column derivatization, cannot meet the requirement of the simultaneous determination of 1–4 in biological fluids. Taking account of the separation conditions for 1–4 in human urine, as previously reported[25], HPLC conditions were explored for routine assays

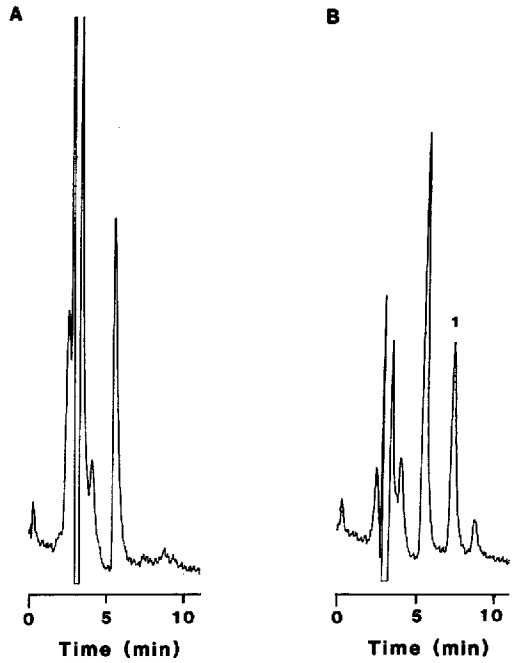

Fig. 3. Chromatogram of 1 in rat plasma. (A) Pre-dose and (B) 60-min post-dose plasma samples were treated as described under Experimental. A 20-μl portion of the sample was loaded on to the column. Detector sensitivity, 0.016 a.u.f.s. For other HPLC and post-column reaction conditions, see Experimental. Peak 1 is 1. The concentration of 1 was 19.5 μg/ml.

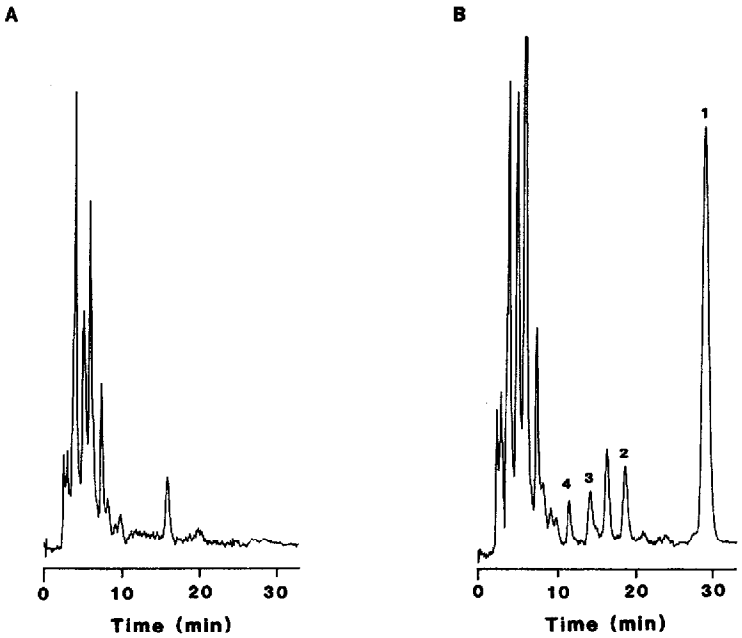

Fig. 4. Chromatogram of 1 and its metabolites in rat bile. After 5-fold dilution, (A) pre-dose and (B) 2–4-h post-dose bile samples were treated as described under Experimental. A 20-μl portion of the sample was loaded on to the column. Detector sensitivity, 0.032 a.u.f.s. (0–25 min) and 0.256 a.u.f.s. (from 25 min). For other HPLC and post-column reaction conditions, see Experimental. Peaks 1, 2, 3 and 4 are compounds 1, 2, 3 and 4, respectively. Concentrations: 1, 87,0 μg/ml; 2, 11.7 μg/ml; 3, 4.8 μg/ml; 4, 2.6 μg/ml.

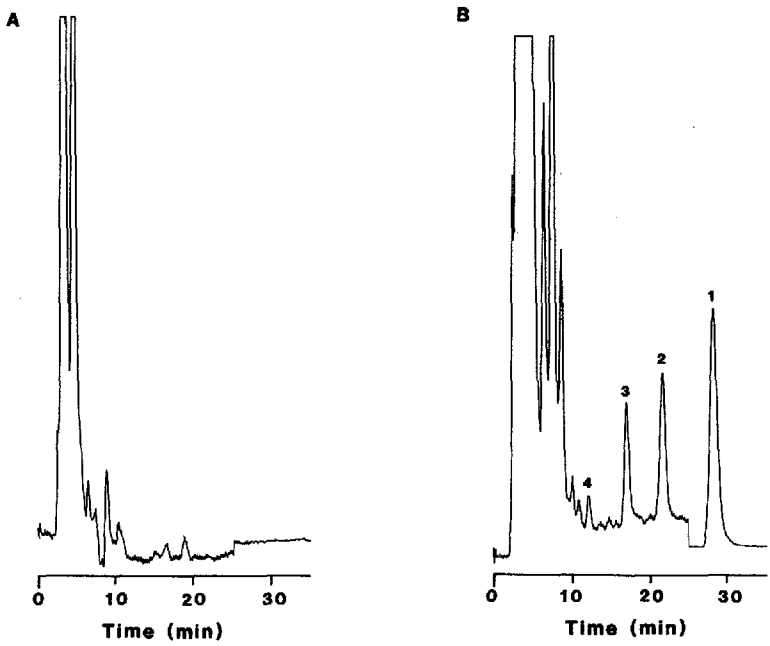

Fig. 5. Chromatogram of 1 and its metabolites in rat urine. After 10-fold dilution, (A) pre-dose and (B) 2–4-h post-dose urine samples were treated as described under Experimental. A 20-μl portion of the sample was loaded on to the column. Detector sensitivity, 0.032 a.u.f.s. (0–25 min) and 0.64 a.u.f.s. (from 25 min). For other HPLC and post-column reaction conditions, see Experimental. Peaks as in Fig. 5. Concentrations: 1, 200 μg/ml; 2, 23.7 μg/ml; 3, 13.4 μg/ml; 4, 2.1 μg/ml.

of rat plasma, bile and urine, and those described under Experimental were finally obtained. As the plasma levels of 2–4 after administration of 1 (100 mg/kg) were below the detection limit, the assay of 1 in plasma samples was performed with eluent A (which does not contain sodium heptanesulphonate).

An advantage of the proposed method is that it is easy to separate these compounds completely from the background components of plasma, bile and urine, in contrast to detection at 220–230 nm (which is a suitable wavelength for the direct UV detection of 1). A second advantage is that repeated injections can be made, that is, the elution of background components due to the preceding injections does not interfere with subsequent analyses.

Fig. 3 shows a chromatogram of the 60-min plasma after intravenous administration of 1, and Figs. 4 and 5 show chromatograms of the 2–4-h bile and urine after administration of 1. Compounds 1–4 were completely separated from the background components of plasma, bile and urine with no interferences.

Previously, we reported[28] that the epimerization of amoxicillin piperazine-2,5-dione in acidic solutions occurred at the 2-position (which corresponds to the 5-position of the parent penicillin), and that the absolute configuration of amoxicillin piperazine-2,5-dione, excreted in human urine, at the 2-position was *R* and the (2*S*)-epimer was not excreted. It was now found that in the rat the absolute configuration of 4 at the 2-position is also *R* and the (2*S*)-epimer is not excreted.

Recovery

Table I shows the recoveries of 1, 2, 3 and 4 from plasma, bile and urine. The recovery of 2–4 from plasma was greater than 95%.

TABLE I

RECOVERY OF 1 AND ITS METABOLITES FROM PLASMA, BILE AND URINE

Results as means (%) ± S.D. (%) of three determinations. In bile and urine samples, the concentration of each compound was 5.0 or 20.0 μg/ml after 10-fold dilution.

Sample	*Concentration (μg/ml)*	*Recovery (%)*			
		1	2	3	4
Plasma	2.0	103 ± 5.3			
	10.0	97.2 ± 5.1			
Bile	5.0	99.9 ± 1.8	93.9 ± 4.3	92.5 ± 2.7	98.3 ± 2.8
	20.0	97.9 ± 0.8	95.4 ± 1.7	90.0 ± 3.2	98.1 ± 1.4
Urine	5.0	96.2 ± 4.0	102 ± 3.3	96.2 ± 4.0	98.0 ± 3.5
	20.0	97.2 ± 2.6	100 ± 3.4	95.0 ± 3.0	101 ± 2.4

Linearity and detection limit

Five- to seven-point calibration graphs for each compound (with concentration ranges as described under Experimental) were linear and passed through the origin with a correlation coefficient above 0.99. The detection limit was about 25 ng for each compound at a signal-to-noise ratio of 3.

Precision

Tables II, III and IV show the within- and between-run precisions for the assays of 1, 2, 3 and 4 in plasma, bile, and urine, respectively.

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OF 1 IN PLASMA

The concentration of 1 present was 2.0 μg/ml.

Assay	*Concentration of 1 found (%)*
1*	2.00 ± 7.07
2*	2.02 ± 5.42
3*	1.94 ± 4.61
Between-run**	1.99 ± 2.10

* Mean (μg/ml) ± coefficient of variation (C.V.) (%) of five determinations.

** Mean (μg/ml) ± C.V. (%) of three determinations.

TABLE III

ACCURACY AND PRECISION OF THE ASSAY OF 1 AND ITS METABOLITES IN BILE

The concentration of each compound present was 5.0 μg/ml in 10-fold diluted bile.

Assay	*Concentration found (%)*			
	1	2	3	4
1*	5.10 ± 4.07	4.66 ± 3.11	4.72 ± 3.95	5.05 ± 4.64
2*	4.87 ± 0.77	5.20 ± 2.80	5.10 ± 3.20	4.88 ± 2.83
3*	5.00 ± 2.18	5.15 ± 4.80	5.25 ± 4.57	5.00 ± 4.13
Between-run**	4.99 ± 2.31	5.00 ± 5.96	5.02 ± 5.44	4.98 ± 1.76

* Mean (μg/ml) ± C.V. (%) of five determinations.
** Mean (μg/ml) ± C.V. (%) of three determinations.

Pharmacokinetics

Compound 1 (100 mg/kg) was administered intravenously to three rats, and the plasma, biliary and urinary levels of 1, 2, 3 and 4 were determined by the proposed HPLC method. The plasma levels of 2–4 were <0.5 μg/ml (which is the limit of detection). Fig. 6 shows the mean plasma concentration–time curve for 1. The results for AUC, MRT, CL_T and Vss and f_{bil} and f_e for each species are given in Table V. It was found that after intravenous administration of 1, 75.6% of the dose was excreted in the bile and urine at infinite time, 69.5% in the unchanged form and 6.1% as the metabolites. The excretion data obtained were consistent with those reported by Tsuji *et al.*[29], except that the amount of the metabolites excreted via the bile was about one-third. The amounts of metabolites reported by Tsuji *et al.* were the total amounts of penicilloic acids [obtained by subtracting the amount of unchanged penicillin from that of total penicillin (unchanged form plus penicilloic acids)]. Hence the large difference in biliary excretion of metabolites might be due to the presence of another metabolite(s) in bile or an overestimate of the amount of penicilloic acids.

TABLE IV

ACCURACY AND PRECISION OF THE ASSAY OF 1 AND ITS METABOLITES IN URINE

The concentration of each compound present was 5.0 μg/ml in 10-fold diluted urine.

Assay	*Concentration found (%)*			
	1	2	3	4
1*	4.96 ± 1.85	4.95 ± 4.70	5.10 ± 7.15	4.93 ± 2.99
2*	5.10 ± 4.00	4.95 ± 2.51	4.84 ± 6.13	4.93 ± 5.72
3*	4.88 ± 5.48	5.15 ± 4.81	4.84 ± 4.53	4.71 ± 5.10
Between-run**	4.95 ± 2.63	5.02 ± 2.30	4.93 ± 3.05	4.86 ± 2.62

* Mean (μg/ml) ± C.V. (%) of five determinations.
** Mean (μg/ml) ± C.V. (%) of three determinations.

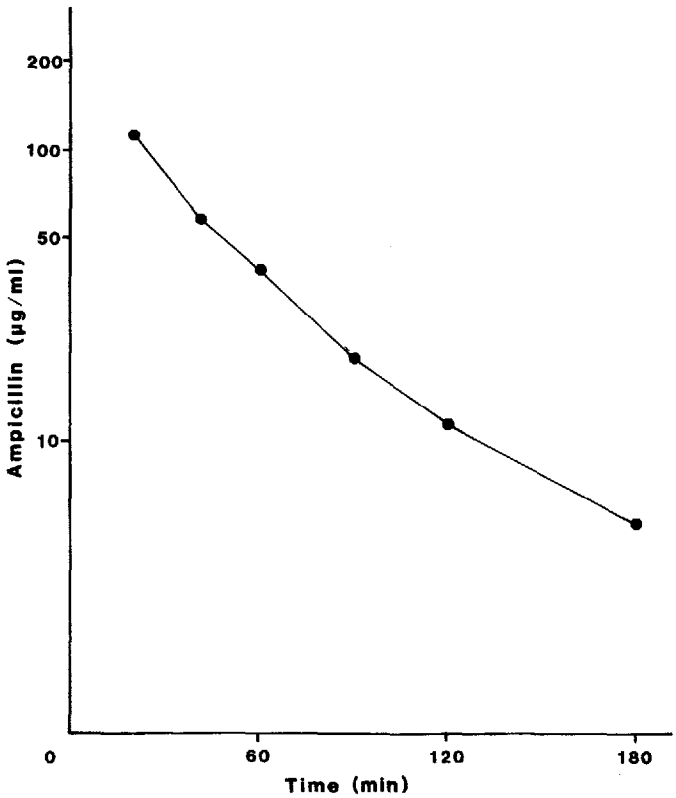

Fig. 6. Mean semi-logarithmic plot of plasma concentration of 1.

This is the first report dealing with the excretion of individual ampicillin metabolites in bile and urine.

The proposed HPLC method should be also applicable to the assay of other penicillins and their metabolites in body fluids with slight modifications.

TABLE V

STATISTICAL PARAMETERS FOR 1 AND ITS METABOLITES FOLLOWING INTRAVENOUS ADMINISTRATION OF 1 TO THREE RATS

The values given are means ± S.D.

Parameters	*1*	*2*	*3*	*4*
AUC (mg min/ml)	5.89 ± 2.68			
MRT (min)	59.3 ± 5.7			
Vss (ml/kg)	1130 ± 415			
CL_T (ml/min/kg)	19.4 ± 8.2			
f_{bil} (%)	23.0 ± 3.8	1.96 ± 0.49	0.53 ± 0.20	0.63 ± 0.14
f_e (%)	46.5 ± 2.8	1.76 ± 0.20	0.89 ± 0.47	0.36 ± 0.18

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