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Liquid chromatographic determination of ampicillin and its metabolites in human urine by postcolumn alkaline degradation

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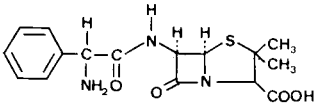
A high-performance liquid chromatographic method has been developed for the determination of ampicillin (I) and its metabolites ((5*R*,6*R*)-penicilloate (II), the (5*S*,6*R*)-epimer (III), and piperazine-2,5-dione (IV)) in human urine. The assay was based on the measurement of the absorbance at 300 nm following the postcolumn alkaline degradation with 0·75 M sodium hydroxide, 2×10^{-3} M mercuric chloride, and 1×10^{-2} M ethylenediaminetetraacetic acid disodium salt in solution. The limits of accurate determination were 0·5 μg mL^{-1} for I, 2·0 μg mL^{-1} for II and III, and 1·0 μg mL^{-1} for IV in neat urine samples with a 10 μL injection. At concentrations of compounds I–IV of 5 μg mL^{-1}, within- and between-run precisions were 1·10–4·03% and 0·93–2·34%, respectively. The urinary levels of I and its metabolites were quantified by the proposed method.

A number of HPLC methods have been developed for ampicillin (I) (Tsuji & Robertson 1975; Vree et al 1978; Larsen & Bundgaard 1978; Westerlund et al 1979; Bundgaard & Larsen 1979; Nachtmann & Gstrein 1980; Uno et al 1981; White & Zarembo 1981; Margosis 1982; Nakagawa et al 1982; Miyazaki et al 1983; Roets et al 1984; Lauback et al 1984; Rogers et al 1984; Haginaka & Wakai 1985a) in pharmaceutical preparations and in body fluids. Masada et al (1979) reported its HPLC separation, the corresponding (5*R*,6*R*)-penicilloate (II) and a new metabolite in human urine, and identified the new metabolite as the corresponding penamaldate. However, Bird et al (1983) reassigned it as the corresponding (5*S*,6*R*)-penicilloate (III). Recently, it has been reported that piperazine-2,5-dione (IV) is a new metabolite of I in rat (Everett et al 1984) and human (Haginaka & Wakai 1986) urine.

The HPLC detection of a penicillin has been mainly made at 220–254 nm utilizing a weak, native UV absorption. However, the detection at this wavelength is deficient in selectivity and/or sensitivity. We describe an HPLC method with postcolumn alkaline degradation with sodium hydroxide, mercuric chloride and ethylenediaminetetraacetic acid disodium salt (EDTA) in solution for the simultaneous determination of ampicillin and its metabolites (II, III and IV) in human urine. The method was successfully applied following the administration of therapeutic doses of the drug or its prodrug (talampicillin) and can be used in biopharmaceutical and pharmacokinetic studies.



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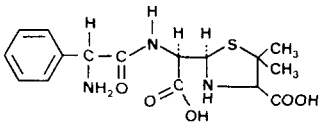

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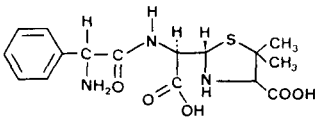

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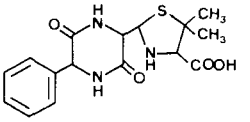

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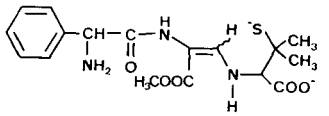
* Correspondence.



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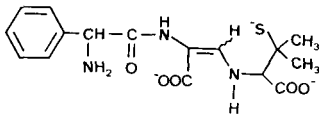

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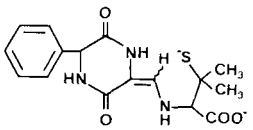

VII

MATERIALS AND METHODS

Materials

Ampicillin (I) standard and capsules of I, and talampicillin (phthalidyl ester of I), were kindly donated by Meiji Seika Kaisha and Beecham Yakuhin (Tokyo, Japan). II and III were prepared according to Bird et al (1983). IV was obtained as previously described (Bundgaard & Larsen 1979). Sodium heptylsulphonate and other chemicals of analytical reagent grade were obtained from Nakarai Chemicals (Kyoto, Japan), and used without further purification. De-ionized, distilled water and distilled methanol were used for the preparations of sample solutions and HPLC eluents.

The HPLC eluent used was 5 mM sodium heptylsulphonate plus 1 mM sodium dihydrogen phosphate and 9 mM phosphoric acid–methanol (1·5 : 1, v/v) (final pH 3·0). The postcolumn reagent used was 0·75 M sodium hydroxide, 2×10^{-3} M mercuric chloride and 1×10^{-2} M EDTA solution.

Apparatus

Fig. 1 depicts a schematic diagram of the instrumentation used in this study. The eluent was delivered through an LC-5A pump (Shimadzu, Kyoto) at a flow rate of 0·8 mL min^{-1}. The stationary phase used was Nucleosil $5C_{18}$ (particle size of 5 μm, Macherey-Nagel, Düren, West Germany) packed in a 4·6 mm i.d. × 15 cm stainless steel column. A sample was loaded onto a column using a variable loop injector (a model 7125, Rheodyne, California, USA). An NP-DX-2 pump (Nihon Seimitsu Kagaku, Tokyo) was used to deliver the postcolumn reagent at a flow rate of 0·2 mL min^{-1}. All flow lines after the column were made with 0·5 mm i.d. Teflon tubing. The

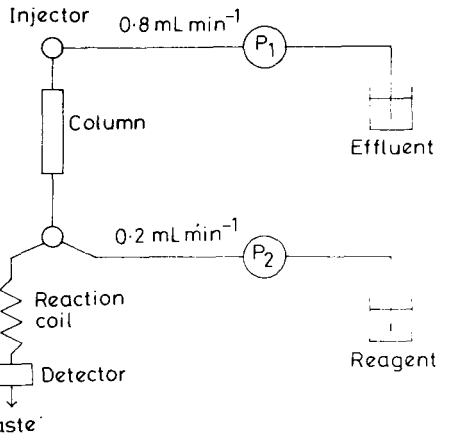

FIG. 1. HPLC flow diagram. P_1 and P_2, pumps.

reagent stream and eluent were mixed in a Diflon T-piece (each angle, 120°). The reaction coil length was 2 m with the residence time of about 24 s. The detector was an SPD-6AV spectrophotometer (Shimadzu, Kyoto) equipped with an 8 μL flow through cell. The detection was performed at 300 nm. All separations and postcolumn reactions were carried out at ambient temperature.

Drug administration

I, or talampicillin (each 500 mg as potency), was orally administered to a human subject. Urine samples were collected at 0, 1, 2, 3, 4, 5, 6, and 8 h after administration. After the urine volume had been measured, the sample was diluted 2- to 5-fold with water, passed through a 0·45 μm acrylate copolymer membrane, and a 10 μL portion of the filtrate loaded onto an HPLC column.

Urine standards of concentration ranges over 2000 to 200 μg mL^{-1} for I, 500 to 50 μg mL^{-1} for II and III, and 100 to 20 μg mL^{-1} for IV were prepared, and diluted 5-fold with water. The standards were passed through the 0·45 μm membrane. Calibration graphs were constructed by plotting peak height versus concentration.

Pharmacokinetics. The percent of dose excreted in urine up to time infinity (f) and the mean residence time (MRTe) were calculated by the method of Yamaoka et al (1978).

RESULTS AND DISCUSSION

HPLC separation

In a previous report (Haginaka & Wakai 1985b), we described that I, II and III were separated on a C_{18} column by the use of phosphate buffer (pH 4·2)–methanol (3 : 1, v/v) as an eluent. However, in the eluent system the separation of II and III from the

ordinary components of urine was not attained, while IV was well retained on the C_{18} column ($k' > 20$) compared with I, II and III, due to its hydrophobicity. Thus, it was impossible to separate I, II, III and IV from the ordinary components of urine in a practical time using phosphate buffer–methanol as an eluent. The separation of I, II, III and IV from the background components of urine was examined by using sodium heptylsulphonate as an anionic ion-pairing agent. Thus, the HPLC conditions described under Materials and methods were selected for the assay of these compounds.

Previously we observed that the addition of methanol to a postcolumn reagent resulted in reduction of the noise levels (Haginaka & Wakai 1985b). However, it was found that the exclusion of methanol from the postcolumn reagent was superior to the addition of methanol in terms of accuracy and precision. Thus, methanol was omitted from the postcolumn reagent.

Structure of alkaline degradation products

In a previous paper (Haginaka & Wakai 1985b), we described how I can be detected as the enamine form of the methyl penamaldate (V), and II and III as the enamine form of the penamaldate (VI). Bundgaard & Larsen (1979) reported that reaction of IV with mercuric chloride in solution at pH 7·0 gave the corresponding penamaldate (VII) (which had a UV absorption maximum at 305 nm). IV can be also detected as VII in this postcolumn reaction system.

HPLC assay

Under the optimal postcolumn and HPLC conditions mentioned above, the 2–3 h urine orally dosed with talampicillin was diluted 5-fold with water, and 10 μL of the diluted urine was loaded onto the HPLC column. As shown in Fig. 2, I, II, III and IV were well separated from the background components of urine. A calibration graph of each compound was linear and passed through the origin with the correlation coefficient above 0·99. The limits of accurate determination were 0·5 μg mL^{-1} for I, 2·0 μg mL^{-1} for II and III, and 1·0 μg mL^{-1} for IV in neat urine samples (with no dilution and a 10 μL injection). Table 1 shows the within- and between-run precisions at concentrations of compounds I–IV of 5 μg mL^{-1} in 5-fold diluted urine. The coefficients of variation of within- and between-run were 1·10–4·03% (n = 5) and 0·93–2·34% (n = 3), respectively.

Urinary excretions of I and its metabolites

I or talampicillin was administered to a human subject, and the urinary levels of I, II, III and IV were determined by the established HPLC method. Figs 3 and 4, where the values are given as equivalent to I, show the time courses of urinary excretion rates of I, II, III and IV after administration of I and talampicillin. The results for the f and MRTe for

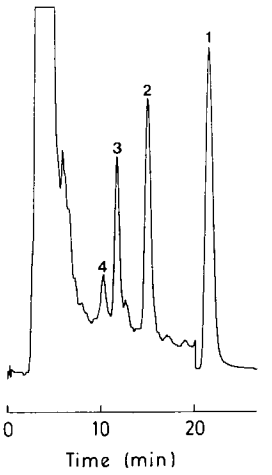

FIG. 2. Separation of ampicillin and its metabolites from the background components of urine. The 2–3 h urine after administration of talampicillin (500 mg as potency) was diluted 5-fold with water and a 10 μL portion of the diluted urine was loaded onto an HPLC column. Key: 1, ampicillin (I); 2, (5*R*,6*R*)-penicilloate (II); 3, (5*S*,6*R*)-penicilloate (III); 4, piperazine-2,5-dione (IV). Sensitivity: 0·032 a.u.f.s., 0–20 min; 0·512 a.u.f.s., from 20 min. The concentration of I was estimated to be 892 μg mL^{-1}; II, 276 μg mL^{-1}; III, 97·2 μg mL^{-1}; IV, 13·2 μg mL^{-1} in 5-fold diluted urine.

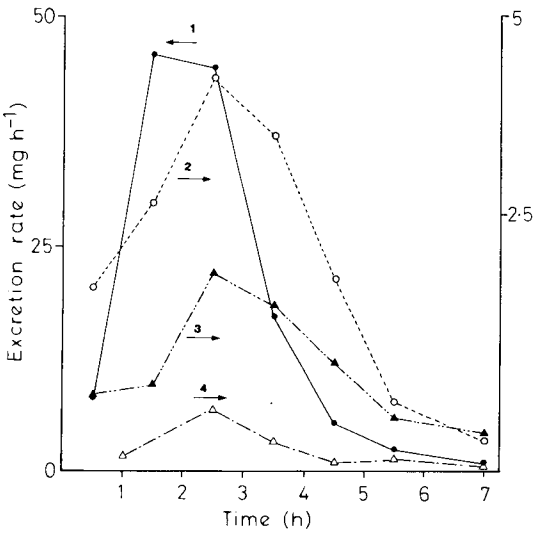

FIG. 3. Urinary excretion rates of ampicillin and its metabolites after administration of ampicillin. Key: 1, ampicillin (I) (the left ordinate); 2: (5*R*,6*R*)-penicilloate (II) (the right ordinate); 3, (5*S*,6*R*)-penicilloate (III) (the right ordinate); 4, piperazine-2,5-dione (IV) (the right ordinate).

Table 1. Accuracy and precision of the assay of ampicillin and its metabolites.[a]

I	II	III	IV
5·04 ± 1·10[b]	4·85 ± 3·49[b]	5·05 ± 4·03[b]	5·00 ± 2·38[b]
4·94 ± 1·13[b]	5·08 ± 3·12[b]	5·00 ± 2·25[b]	4·91 ± 1·58[b]
5·06 ± 2·98[b]	5·00 ± 3·39[b]	4·92 ± 1·50[b]	4·94 ± 3·15[b]
Between run			
5·01 ± 1·28[c]	4·98 ± 2·34[c]	4·99 ± 1·31[c]	4·95 ± 0·93[c]

[a] The concentration of each compound was 5·00 μg mL^{-1} in 5-fold diluted urine.
[b] Mean (μg mL^{-1}) ± CV (coefficient of variation) of five determinations.
[c] Mean ± CV of three determinations.

Table 2. Statistical moments for urinary excretion of ampicillin and its metabolites following oral administration of ampicillin or talampicillin (each 500 mg as potency).

	I	II	III	IV
Ampicillin				
f[a] (%)	25·1	3·29	1·82	0·37
MRTe[b] (h)	2·37	2·99	3·78	3·45
Talampicillin				
f (%)	58·1	10·6	4·22	0·86
MRTe (h)	2·13	3·19	3·47	3·02

[a] f is the fraction of dose excreted in urine.
[b] MRTe is the mean residence time from administration to urinary excretion.

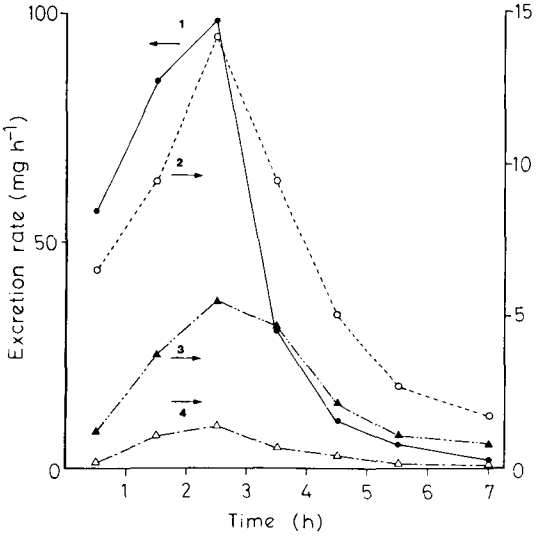

FIG. 4. Urinary excretion rates of ampicillin and its metabolites after administration of talampicillin. Key is the same as that for Fig. 3.

each species are given in Table 2. It was found that after administration of I, 30·6% of the dose was excreted in urine at infinite time, 25·1% as the unchanged form, and 5·5% as the metabolites; after administration of talampicillin, 73·1% as the total, 58·1% as the unchanged form, and 15·7% as the metabolites. The MRTe values after the administration of I were almost consistent with those of talampicillin. These results reveal that the total excretions of talampicillin are 2·4 times larger than those of I, but that there are no significant differences in the rate process among I and talampicillin.

The proposed HPLC method may well be useful for the assay of other penicillins and their metabolites in urine and plasma with a slight modification.

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