Journal of Chromatography, 413 (1987) 219–226
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3368

LIQUID CHROMATOGRAPHIC DETERMINATION OF AMOXICILLIN AND ITS METABOLITES IN HUMAN URINE BY POSTCOLUMN DEGRADATION WITH SODIUM HYPOCHLORITE

JUN HAGINAKA* and JUNKO WAKAI

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16 Edagawa-cho, Nishinomiya, Hyogo 663 (Japan)

(First received June 2nd, 1986; revised manuscript received July 29th, 1986)

SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of amoxicillin (I) and its metabolites [(5*R*,6*R*)-amoxicilloic acid (II), the (5*S*,6*R*) epimer (III), and the (2*R*)-piperazine-2′,5′-dione (IV)] in human urine. They were separated from the background components of urine on a reversed-phase C_{18} column using sodium heptylsulphonate as an ion-pairing agent and methanol as an organic mobile phase modifier. The eluent was led to the postcolumn degradation with 1.5 *M* sodium hydroxide plus 0.02% sodium hypochlorite solution at ambient temperature. The degradation product(s) of each compound was detected at 270 nm. The proposed method permits detection of I, II, III, and IV down to 1 μg/ml in neat urine samples. At a concentration of 5 μg/ml of each compound, within- and between-run precisions (relative standard deviation) were 1.12–5.79 and 0.80–2.70%, respectively. The urinary levels of I and its metabolites were determined by the proposed method after administration of I to humans.

INTRODUCTION

Amoxicillin (I) is an orally absorbed, acid-stable, broad-spectrum antimicrobial agent. The assay of this drug in pharmaceutical preparations and in body fluids by high-performance liquid chromatography (HPLC) has been mainly based on direct UV detection at ca. 230 nm [1–12]. The separation of I from the background components of body fluids is laborious with the detection at such a wavelength. In order to enhance selectivity and sensitivity, several HPLC methods combined with precolumn [13,14] and postcolumn [15–17] reactions have been developed for the assay of I in body fluids.

It is well known that penicillins are metbolized in humans to the corresponding (5*R*,6*R*)-penicilloic acids and the (5*S*,6*R*) epimers [18]. In a previous paper [19], we reported that ampicillin piperazine-2,5-dione is excreted as a metabolite

0378-4347/87/$03.50

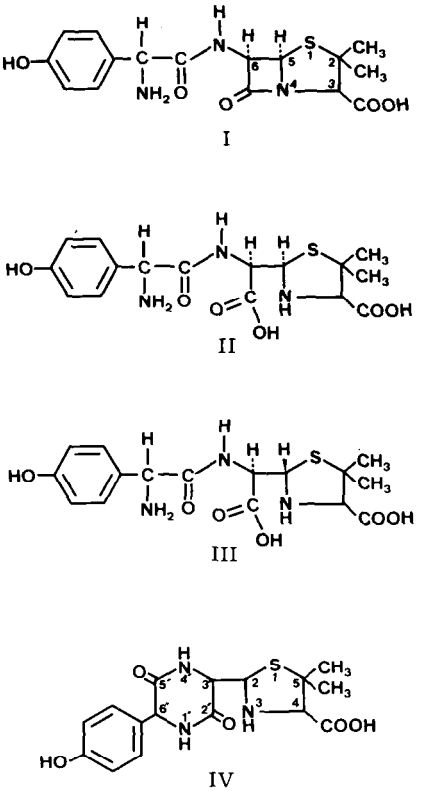

Fig. 1. Structures of amoxicillin and its metabolites: I = amoxicillin; II = (5*R*,6*R*)-amoxicilloic acid; III = (5*S*,6*R*)-amoxicilloic acid; IV = (2*R*)-piperazine-2′,5′-dione.

in human urine after administration of ampicillin. Preliminary examination of the human urine dosed with I also showed the excretion of amoxicillin piperazine-2′,5′-dione (IV) in addition to I, (5*R*,6*R*)-amoxicilloic acid (II) and the (5*S*,6*R*) epimer (III). Previously we reported [20] an HPLC method for the determination of penicillins using the postcolumn reaction with sodium hypochlorite. The present paper deals with the application of the method to the simultaneous determination of I and its metabolites (II, III and IV) in human urine, and pharmacokinetic studies following the administration of therapeutic dose of I.

EXPERIMENTAL

Materials

The structures of I and its metabolites (II, III and IV) are shown in Fig. 1. Compounds II and III were prepared by methods similar to those reported previously [3,18] and IV was prepared according to the procedures reported previously [21]. Other chemicals of reagent grade were purchased from Nakarai (Kyoto, Japan) and used without further purification. Deionized, glass-distilled water and distilled methanol were used to prepare the HPLC eluents.

Chromatography

The following HPLC system was used: two pumps, Trirotar-V (Japan Spectroscopic Co., Tokyo, Japan) and NP-DX-2 (Nihon Seimitsu Kagaku, Tokyo, Japan), for delivering the eluent and postcolumn reagent, respectively; a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 100-μl loop for the loading of the samples; and a Uvidec-100-V (Japan Spectroscopic Co.) spectrophotometer equipped with an 8-μl cell for detection.

Chemically C_{18}-bonded silica (Nucleosil $5C_{18}$, 5-μm particle size, Macherey-Nagel, Düren, F.R.G.) was used, packed in a 15 cm$\times$4.6 mm I.D. stainless steel column. The mobile phase used was 15 m*M* sodium heptylsulphonate–12 m*M* sodium dihydrogen phosphate–18 m*M* phosphoric acid–methanol (1:1:1:1.2). The flow-rate was maintained at 0.8 ml/min. The postcolumn reagent used was 1.5 *M* sodium hydroxide plus 0.02% sodium hypochlorite solution, at a flow-rate of 0.2 ml/min. PTFE tubing (1 m$\times$0.5 mm I.D.) was used as the postcolumn reactor. The reagent stream and eluent were mixed in a Diflon tee-piece (each angle, 120°). Detection was performed at 270 nm. All separations and postcolumn reactions were carried out at ambient temperature.

Calibration graph

Urine standards of concentration ranges over 2000–200 μg/ml for I, 500–50 μg/ml for II and III and 100–20 μg/ml for IV in neat urine samples were prepared and diluted ten-fold with water. The diluted urine sample was passed through a 0.45-μm acrylate copolymer membrane and a 20-μl portion of the filtrate was loaded onto an HPLC column. Calibration graphs were constructed by plotting peak height versus concentration.

Drug administration

Compound I (500 mg as potency) was orally administered to three human subjects fasted for 12 h. Urine samples were collected at 0, 1, 2, 3, 4, 6 and 8 h after administration. The volume of a sample was measured, and it was then diluted two- to twenty-fold with water, and stored at −80°C until assay. The diluted urine sample was treated as described above.

Pharmacokinetics

In pharmacokinetic studies, the zero moment for urinary excretion data is the total amount of a drug excreted up to time infinity ($Ae(\infty)$); and the first moment, the mean residence time ($MRTe$). These moments are defined as follows:

$$Ae(\infty) = \int_0^\infty (\mathrm{d}Ae/\mathrm{d}t)\,\mathrm{d}t \tag{1}$$

$$MRTe = \int_0^\infty t(\mathrm{d}Ae/\mathrm{d}t)\,\mathrm{d}t \Big/ \int_0^\infty (\mathrm{d}Ae/\mathrm{d}t)\,\mathrm{d}t \tag{2}$$

where $\mathrm{d}Ae/\mathrm{d}t$ is urinary excretion rate [22]. They were calculated by trapezoidal integration with extrapolation of time-course curves to infinite time using a mono-

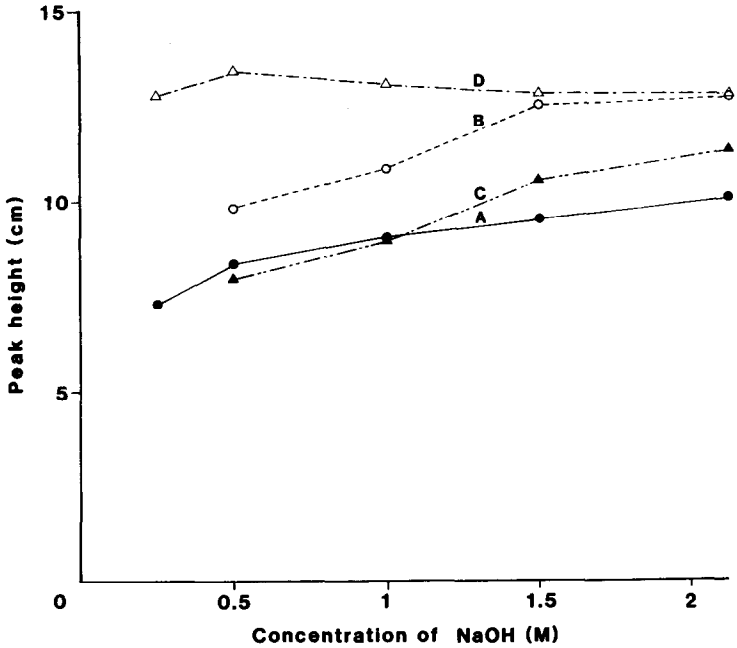

Fig. 2. Effect of sodium hydroxide concentration on the UV absorbance of the degradation products of I, II, III and IV determined by flow injection analysis. The concentration of sodium hypochlorite was fixed at 0.02%. Curves: A = I; B = II; C = III; D = IV. A 20-μl portion of each sample solution (25 μg/ml) was injected at a detector sensitivity of 0.16 a.u.f.s.

exponential equation. The percentage of the dose excreted in urine up to time infinity (f) is given by

$$f = Ae(\infty)/D \times 100 \quad (3)$$

where D is the dose.

RESULTS AND DISCUSSION

Postcolumn reaction conditions

The postcolumn reaction conditions (pH, reagent concentration) were examined to study their effects on the UV absorbance produced by the reaction of I, II, III and IV with sodium hypochlorite using the flow injection analysis. The carrier solution (eluent for HPLC) and postcolumn reagent were delivered at flow-rates of 0.8 and 0.2 ml/min, respectively, and the reaction coil length was fixed at 1 m (corresponding to 12 s of the residence time). Fig. 2 shows the effect of changing the concentration of sodium hydroxide solution on the UV response at a sodium hypochlorite concentration of 0.02%. The maximum UV response was obtained at a sodium hydroxide concentration of 0.5 M for IV, but a gradual increase of the response was observed with I, II and III as the sodium hydroxide concentration was raised. Fig. 3 shows the effect of changing the concentrations of sodium hypochlorite from 0.005 to 0.04% at sodium hydroxide concentrations

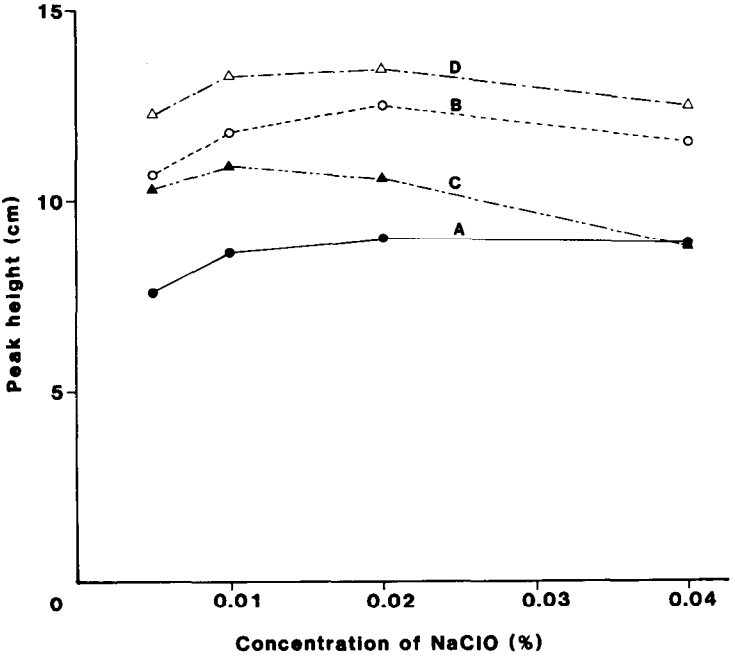

Fig. 3. Effect of sodium hypochlorite concentration on the UV absorbance of the degradation products of I, II, III and IV determined by flow injection analysis. The concentration of sodium hydroxide solution was 1.0 *M* for I, 1.5 *M* for II and III and 0.5 *M* for IV. Curves: A = I; B = II; C = III; D = IV. A 20-μl portion of each sample solution (25 μg/ml) was injected at a detector sensitivity of 0.16 a.u.f.s.

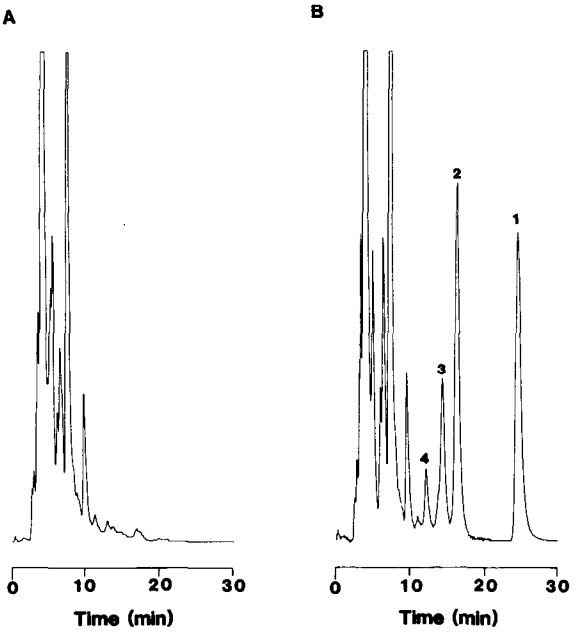

Fig. 4. Chromatogram of I and its metabolites in human urine. A pre-dose urine sample (A) and a 2–3 h post-dose urine sample (B) were diluted twenty-fold with water. A 20-μl portion of the diluted urinesample was loaded onto a column after filtration. Detection was performed at 270 nm and at a detector sensitivity of 0.032 a.u.f.s. (0–21 min) and 0.256 a.u.f.s. (from 21 min). For other postcolumn and HPLC conditions, see Experimental. Peaks 1, 2, 3 and 4 are I, II, III and IV, respectively. The concentration of I was 269 μg/ml in twenty-fold diluted urine; II, 35.9 μg/ml; III, 12.8 μg/ml; and IV, 4.9 μg/ml.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF I AND ITS METABOLITES

The concentration of each compound was 5.0 μg/ml in ten-fold diluted urine. The coefficients of variation (%) are given in parentheses.

Assay No.	Concentration found (mean ± S.D.) (μg/ml)			
	I	II	III	IV
Within-run (n=5)				
1	5.16 ± 0.10 (1.91)	5.05 ± 0.29 (5.69)	5.07 ± 0.21 (4.16)	5.11 ± 0.20 (3.86)
2	5.00 ± 0.06 (1.14)	5.16 ± 0.22 (4.24)	5.05 ± 0.29 (5.79)	5.04 ± 0.21 (4.10)
3	4.92 ± 0.08 (1.64)	4.89 ± 0.13 (2.57)	4.97 ± 0.06 (1.12)	5.04 ± 0.10 (1.95)
Between-run (n=3)	5.03 ± 0.12 (2.43)	5.03 ± 0.14 (2.70)	5.03 ± 0.05 (1.05)	5.06 ± 0.04 (0.80)

of 1.0 *M* for I, 1.5 *M* for II and III, and 0.5 *M* for IV. The maximum UV response was obtained at a sodium hypochlorite concentration of 0.02% for all compounds. Thus, the postcolumn reaction conditions (1.5 *M* sodium hydroxide and 0.02% sodium hypochlorite solution) were selected for the simultaneous determination of I, II, III and IV. The detection wavelength was examined by changing the wavelength of the HPLC detector. The optimal detection wavelengths were 246–248 nm for I–IV. However, with detection at this wavelength the background components of urine interfered the assay of II, III and/or IV. Therefore, detection was performed at 270 nm, where I–IV were ca. four fifths to two fifths as sensitive as at 250 nm.

HPLC separation and detection of I and its metabolites

Several HPLC methods combined with a postcolumn reaction with imidazole and mercury(II) chloride [16], or fluorescamine [15,17] have been developed for the determination of I and II in urine and plasma. However, with these methods it is not possible to determine I, II, III and IV simultaneously in body fluids. The goal of the present investigation is to achieve the sensitive and selective assay of I–IV in human urine. Uno et al. [3] reported the separation of I, II and III from the background components of urine on a C_{18} column using sodium heptylsulphonate as an ion-pairing agent. Therefore, we tried to separate I, II, III and IV using the modified eluent reported by Uno et al. [3]. However, it is difficult to separate these compounds completely from the background components of urine with detection at 230 nm (which is a suitable wavelength for the direct UV detection of I). Another disadvantage of using this wavelength was that, on repeated injections, the elution of background components due to the preceding injection interfered the assay of II, III and/or IV. Fig. 4 shows the separation of four compounds with postcolumn reaction with sodium hypochlorite (detected at 270 nm). They were completely separated from the background components of urine with no interference. Previously we reported [21] that the epimerization of IV in acidic solutions occurred at the 2-position (which corresponds to the 5-position of the parent penicillin). It is found that the absolute configuration of IV excreted in urine at the 2-position is (2*R*), and that the (2*S*) epimer (which

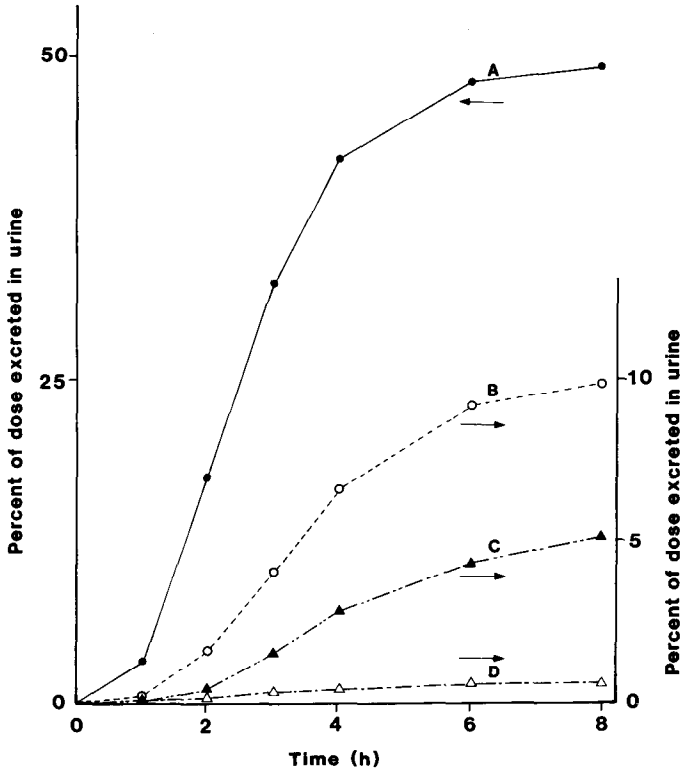

Fig. 5. Cumulative urinary excretion amounts (percentage of dose) of I and its metabolites after administration of I. Curves: A = I; B = II; C = III; D = IV. The values of metabolites are given as equivalent to I.

is eluted at 14 min in this eluent system) is not excreted in urine.

Linearity and precision

A six- to eight-point calibration graph of each compound (over the concentration ranges described under Experimental) was linear and passed through the origin with a correlation coefficient of greater than 0.99. The limits of detection were 1 μg/ml for I, II, III and IV in neat urine samples with a 20-μl injection (after two-fold dilution). Table I shows the within- and between-run precisions.

Urinary excretion of I and its metabolites

Compound I was administered to three human subjects, and the urinary levels of I, II, III and IV were determined by the established HPLC method. Fig. 5, in which the values are given as equivalent to I, shows the time courses of cumulative urinary excretion amounts (percentage of dose) of I, II, III and IV after administration of I. The results for the *f* and *MRTe* values for each species are given in Table II. It was found that after administration of I, 67.5% of the dose was excreted in urine at infinite time, 49.5% as the unchanged form and 18.0% as the metabolites.

TABLE II

STATISTICAL MOMENTS FOR URINARY EXCRETION OF I AND ITS METABOLITES FOLLOWING ORAL ADMINISTRATION OF I TO THREE HUMAN SUBJECTS

f is the fraction of dose excreted in urine, where the values are given as mean ± S.D. of three subjects. *MRTe* is the mean residence time from administration to urinary excretion, where the values are given as mean ± S.D. of three subjects.

Compound	*f* (%)	*MRTe* (h)
I	49.5 ± 5.55	2.72 ± 0.43
II	10.9 ± 0.90	4.13 ± 0.42
III	6.36 ± 2.69	5.22 ± 1.47
IV	0.76 ± 0.33	4.50 ± 1.05

The application of the proposed HPLC method to the assay of other penicillins and their metabolites in body fluids (urine, bile and plasma) is now being investigated.

ACKNOWLEDGEMENTS

The authors thank Drs. T. Uno and H. Yasuda, Mukogawa Women's University, for their support and interest.

REFERENCES

1 T.B. Vree, Y.A. Hekster, A.M. Baars and E. van der Kleijn, J. Chromatogr., 145 (1978) 496.
2 M.J. Lebelle, W.L. Wilson and G. Lauriault, J. Chromatogr., 202 (1980) 144.
3 T. Uno, M. Masada, Y. Yamaoka and T. Nakagawa, Chem. Pharm. Bull., 29 (1981) 1957.
4 J. Haginaka, T. Nakagawa, T. Hoshino, K. Yamaoka and T. Uno, Chem. Pharm. Bull., 29 (1981) 3342.
5 M. Foulstone and C. Reading, Antimicrob. Agents Chemother., 22 (1982) 753.
6 J. Hoogmartens, E. Roets, G. Janssen and H. Vanderhaeghe, J. Chromatogr., 244 (1982) 299.
7 T. Nakagawa, A. Shibukawa and T. Uno, J. Chromatogr., 239 (1982) 695.
8 T.L. Lee and M.A. Brooks, J. Chromatogr., 306 (1984) 429.
9 G.T. Briguglio and C.A. Lau-Cam, J. Assoc. Off. Anal. Chem., 67 (1984) 228.
10 A. Aboul Khier, G. Blaschke and M. El Sadek, Anal. Lett., 17 (1984) 1659.
11 P. de Pourcq, J. Hoebus, E. Roets, J. Hoogmartens and H. Vanderhaeghe, J. Chromatogr., 321 (1985) 441.
12 J.H.G. Jonkman, R. Schoenmaker and J. Hempenius, J. Pharm. Biomed. Anal., 3 (1985) 359.
13 K. Miyazaki, K. Ohtani, K. Sunada and T. Arita, J. Chromatogr., 276 (1983) 478.
14 J. Haginaka and J. Wakai, Analyst (London), 110 (1985) 1277.
15 T.L. Lee, L. D'Arconte and M.A. Brooks, J. Pharm. Sci., 68 (1979) 454.
16 J. Carlqvist and D. Westerlund, J. Chromatogr., 164 (1979) 373.
17 J. Carlqvist and D. Westerlund, J. Chromatogr., 344 (1985) 285.
18 A.E. Bird, E.A. Cutmore, K.R. Jennings and A.C. Marshall, J. Pharm. Pharmacol., 35 (1983) 138.
19 J. Haginaka and J. Wakai, J. Pharm. Pharmacol., 38 (1986) 225.
20 J. Haginaka and J. Wakai, Anal. Chem., 58 (1986) 1896.
21 J. Haginaka and J. Wakai, Chem. Pharm. Bull., 34 (1986) 2239.
22 K. Yamaoka, T. Nakagawa and T. Uno, J. Pharmacokin. Biopharm., 6 (1978) 547.