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LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLINS BY POSTCOLUMN DEGRADATION WITH SODIUM HYPOCHLORITE USING AN HOLLOW-FIBRE MEMBRANE REACTOR

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SUMMARY

A sensitive, high-performance liquid chromatographic method involving postcolumn degradation with sodium hypochlorite and using a hollow-fibre membrane as a reactor is described for the determination of penicillins. Penicillins were separated on a C_{18} column followed by postcolumn reaction with sodium hypochlorite and sodium hydroxide using aminated and sulphonated hollow-fibre membrane reactors immersed in each solution, and detected at 270–280 nm based on the UV absorbances of the degradation products. At penicillin concentrations of 2 μg/ml, the precisions (relative standard deviation) were 2.28–4.78%. The detection limits of the proposed method were 2.5–25 ng for each penicillin at a signal-to-noise ratio of 3. Ampicillin and its metabolites [(5*R*,6*R*)-ampicilloic acid, the (5*S*,6*R*)-epimer and (2*R*)-pierazine-2′,5′-dione] in human serum and urine were simultaneously determined by this method.

INTRODUCTION

Some strategies for the sensitive and selective determination of a drug and its metabolite(s) in body fluids by high-performance liquid chromatography (HPLC) are to perform the sample preparation (which includes enrichment of compounds of interest and/or removal of interfering compounds) and/or to develop a precolumn or postcolumn derivatization method. In previous papers, we developed two HPLC methods for the determination of penicillins and their metabolites in plasma and urine which employed postcolumn reactions with (a) sodium hydroxide, mercury(II) chloride and ethylenediaminetetraacetic acid[1,2], and (b) sodium hypochlorite and sodium hydroxide[3,4]. These methods were sensitive to the metabolites as well as the unchanged penicillins, in contrast to previous postcolumn derivatization methods[5–12] which are sensitive only to the unchanged penicillins. Method (b) has the advantage that it does not require the use of mercury(II) chloride, which is toxic to humans and is an environmental pollutant.

The conventional postcolumn reaction method needs an additional pump(s)

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for delivering the reagent solutions, a mixing unit(s) and a reactor(s). The postcolumn reaction method using an hollow-fibre membrane reactor (HFMR) need not employ the additional reaction devices described above. Previously[13–16], we reported that the HFMR is useful for the postcolumn reaction.

This paper deals with an HPLC method for the determination of penicillins using aminated and sulphonated HFMRs to introduce hypochlorite and hydroxide ions into the main flow stream for the postcolumn degradation reaction. The method was successfully applied to the determination of ampicillin and its metabolites in human serum and urine.

EXPERIMENTAL

Reagents and materials

Ampicillin (ABPC), phenethicillin (PEPC), phenoxymethylpenicillin (PCV) and ciclacillin (ACPC) were kindly donated by Meiji Seika Kaisha (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan). PEPC is a mixture of the (10*R*)- and (10*S*)-epimers. Benzylpenicillin (PCG) was obtained from Sigma (St. Louis, MO, U.S.A.). ABPC metabolites [(5*R*,6*R*)-ampicilloic acid (1), the (5*S*,6*R*)-epimer (2) and (2*R*)-piperazine-2′,5′-dione (3)] were prepared according to the methods reported previously[17,18]. The structures and abbreviations of the penicillins and ABPC metabolites used are shown in Figs. 1 and 2, respectively. Sodium heptanesulphonate and other chemicals of analytical reagent grade were obtained from Nakarai Chemicals

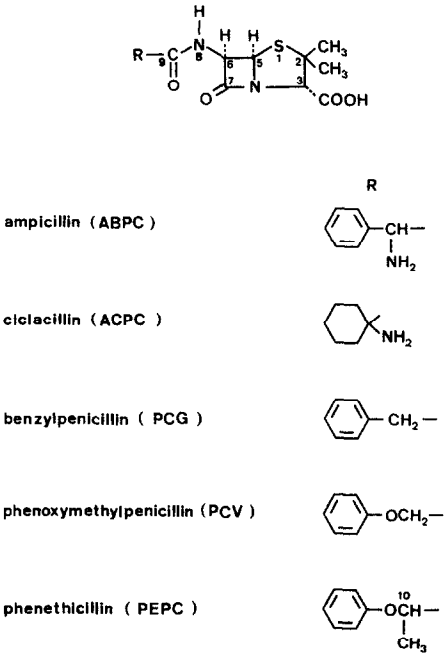

Fig. 1. Structures and abbreviations of penicillins.

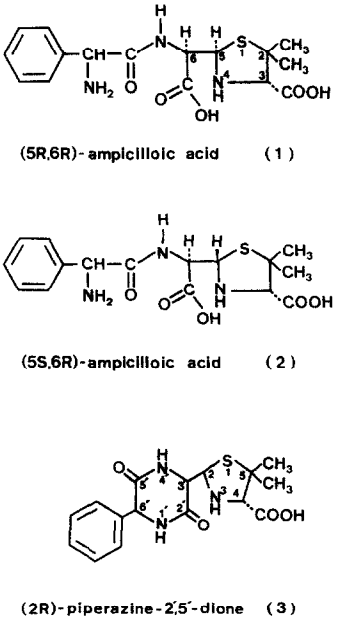

Fig. 2. Structures of ampicillin metabolites.

(Kyoto, Japan). Control serum (Control Serum I Wako) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Sulphonated and aminated hollow-fibre membranes (AFS-2 and CFS1-2 fibres) were obtained from Dionex (Sunnyvale, CA, U.S.A.).

Deionized, glass-distilled water and distilled methanol were used for the preparations of sample solutions and HPLC eluents.

Chromatography

The instrumentation used was as follows: a Model 655 pump (Hitachi, Tokyo, Japan) for eluent delivery; a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A. equipped with a 100-μl loop for sample loading; a main column (125 mm × 4 mm I.D.) and a guard column (4 mm × 4 mm I.D.) packed with LiChrosphere RP-18(e) (particle size 5 μm; E. Merck, Darmstadt, F.R.G.) for the separation of penicillins; a main column (150 mm × 4.6 mm I.D.) and a precolumn (30 mm × 4.6 mm I.D.) packed with Nucleosil C_{18} (particle size 5 μm; Macherey-Nagel, Düren, F.R.G.) for the separation of ABPC and its metabolites; a Model 638-41 variable-wavelength UV monitor (Hitachi) equipped with a 17-μl flow-through cell for detection; a C-R3A recorder-integrator (Shimadzu, Kyoto, Japan) for recording and integrating chromatographic peaks. The eluents used were: A, 10 m*M* sodium dihydrogenphosphate–10 m*M* disodium hydrogenphosphate–methanol (0.9:0.9:1, v/v/v); B, 20 m*M* sodium dihydrogenphosphate–20 m*M* disodium hydrogenphosphate –acetonitrile (3:3:1, v/v/v); C, 15 m*M* sodium heptanesulphonate –21 m*M* phosphoric acid–9 m*M* sodium dihydrogenphosphate–acetonitrile (1.5:1.5:1.5:1). The flow-rate was 0.8 ml/min. Eluent A was used for the separation of penicillins, B for ABPC in serum samples and C for ABPC and its metabolites in urine samples. The aminated and sulphonated HFMRs were connected in series and inserted between the column and the detector. They were immersed in 50-ml beakers containing sodium hypochlorite plus sodium hydroxide solution and sodium hydroxide solution, respectively. The postcolumn reaction conditions (lengths of the HFMRs and concentrations of the reaction solutions) employed for the assay of penicillins and ABPC metabolites are listed in Table I. Separations and postcolumn reactions were carried out at ambient temperature and 50°C, respectively. Detection was performed at 280 nm for unchanged penicillins and 260 nm for ABPC and its metabolites (1, 2 and 3).

Comparison of detection methods

The detection methods were compared as follows: A, detection at 230 nm

TABLE I

OPTIMUM POSTCOLUMN REACTION CONDITIONS

	Penicillins	*ABPC metabolites*
Aminated HFMR length (cm)	50	20
Sodium hypochlorite concentration (%)*	0.2	0.2
Sulphonated HFMR length (cm)	80	80
Sodium hydroxide concentration (*M*)	0.5	3

* Including 1 *M* sodium hydroxide.

without a reactor; B, detection at 280 nm with an open-tubular reactor; C, detection at 280 nm with aminated and sulphonated HFMRs. For method B, the additional reaction devices used were: a double plunger pump (NP-DX-2; Nihon Seimitu Kagaku, Tokyo, Japan) for delivering the postcolumn reagent (0.02% sodium hypochlorite and 0.5 *M* sodium hydroxide) at a flow-rate of 0.2 ml/min; a mixing tee made of Diflon (each angle, 120°) and a reaction coil of 1 m × 0.5 mm I.D. PTFE tube for the postcolumn reaction[3,4]. For method C, the postcolumn reaction conditions were the same as described above.

Pretreatment of serum and urine samples

Serum samples (200 μl) were ultrafiltered using a Molcut II (Nihon Millipore, Tokyo, Japan). A 20-μl portion of the ultrafiltrate was loaded onto a column.

Urine samples, diluted 10-fold in water, were filtered with a 0.45-μm acrylate copolymer membrane (Gelman Science Japan, Tokyo, Japan). A 20-μl portion of the filtrate was loaded onto a column.

RESULTS AND DISCUSSION

Reaction conditions for HFMR

In this study, we attempted to perform the previously reported[3,4] postcolumn reaction by using the HFMRs immersed in sodium hypochlorite and sodium hydroxide solutions. The postcolumn reaction conditions for penicillins, and ABPC and its metabolites, were examined with regard to the lengths of the HFMRs, concentrations of sodium hypochlorite and sodium hydroxide and reaction temperature. Eluents A (for penicillins) and C (for ABPC and its metabolites) were delivered at a flow-rate of 0.8 ml/min. A 20-μl portion of the solution of penicillins or ABPC and its metabolites was loaded onto the column and the peak heights were measured. First the aminated HFMR immersed in sodium hypochlorite plus sodium hydroxide solution was used for introducing hypochlorite and hydroxide ions into the main stream. The UV response obtained was about one fourth to one eighth of that obtained with the conventional postcolumn reaction method reported previously[3]. This is due to an insufficient hydroxide ion concentration for the degradation reaction. Next, the aminated and sulphonated HFMRs were used in series for introducing hypochlorite and hydroxide ions: when the sulphonated HFMR was followed by the aminated one, the UV response was scarcely obtained; on the contrary, when the aminated HFMR was followed by the sulphonated one, the response obtained was about one third of that obtained with the conventional method[3]. At the reaction temperature of 50°C, the peak height obtained was higher than that obtained with the conventional method[3]. When sodium hydroxide was not added to hypochlorite solution, the UV response was hardly obtained. Thus, sodium hydroxide was added to hypochlorite solution at a concentration of 1 *M*.

When an eluent containing acetonitrile as an organic modifier was used, ABPC and its metabolites gave two- to three-times higher UV responses, compared with the use of an eluent containing methanol. Thus, the postcolumn reaction conditions were selected as described under Experimental. The optimum detection wavelength, examined by using the HPLC detector, was 270–280 nm for unchanged penicillins, 255 nm for compounds 1 and 2. Compound 3 had no UV absorption maximum above 230

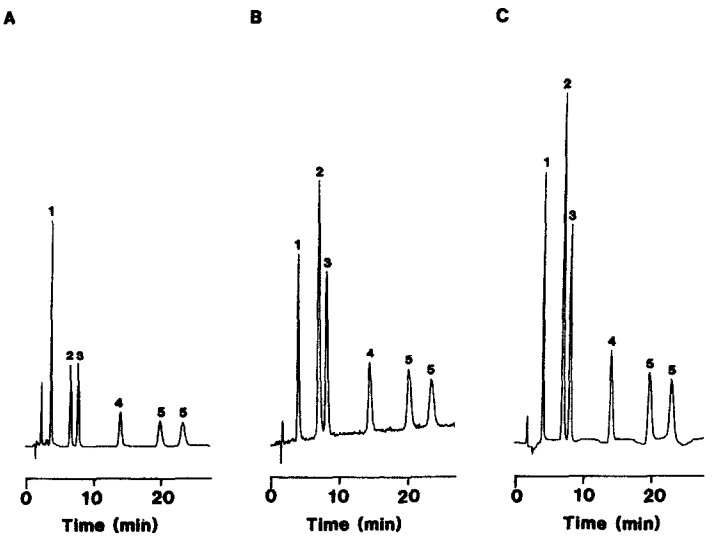

Fig. 3. Comparison of the three detection methods for penicillins: (A) detection at 230 nm without a reactor (method A); (B) detection at 280 nm with an open-tubular reactor (method B); (C) detection at 280 nm with aminated and sulphonated HFMRs (method C). A 20-μl portion of a mixture of ABPC (25 μg/ml), ACPC (25 μg/ml), PCG (25 μg/ml), PCV (25 μg/ml) and PEPC (50 μg/ml) was loaded onto the column. Sensitivity: 0.064 a.u.f.s. Peak assignments: 1 = ABPC; 2 = ACPC; 3 = PCG; 4 = PCV; 5 = (10*R*)- and (10*S*)-epimers of PEPC. Other conditions were given in the text.

nm. The sodium hypochlorite and sodium hydroxide solutions were used for about 10 and 20 h, respectively, without loss of their activity.

Comparison of detection methods

Fig. 3A–C shows the chromatograms of penicillins detected by three different methods (methods A, B and C). Penicillins were more sensitively detected at 280 nm following the postcolumn reaction (methods B and C), compared with direct UV detection at 230 nm (method A); method C gave 1.2- to 4.2-times higher responses than method A. The peak heights of penicillins obtained in method C were 1.2- to 1.5-times higher than in method B, and the resolution between ACPC and PCG (peaks 2 and 3, in Fig. 3) was 1.79 and 2.10 in methods B and C, respectively.

TABLE II

PRECISION OF THE ASSAY OF PENICILLINS

Relative standard deviations (%) of fifteen analyses, are given.

Penicillin	*Concentration (μg/ml)*	
	2.0	*10.0*
ACPC	3.96	2.14
PCG	4.08	2.89
PCV	2.28	1.75
PEPC	4.78	3.14

Reproducibility, linearity and detection limits

Table II lists the precisions [relative standard deviation (R.S.D.)] for measured peak heights of penicillins. The results reveal good reproducibility for all penicillins. Peak heights were found to be scattered at random around a mean value; that is, no trends (constant decrease in peak height with time) were observed. This reveals that the optimum concentrations of hypochlorite and hydroxide ions are maintained in spite of continuous depletion of hypochlorite and hydroxide ions. The calibration graphs of peak height *versus* concentration for each penicillin were linear in the concentration range 0.5–50 μg/ml with a correlation coefficient of ≥ 0.999, and passed through the origin. The detection limits of the proposed method were 2.5–25 ng for each penicillin at a signal-to-noise ratio of 3.

Application to the determination of ABPC and its metabolites in serum and urine

On the basis of the above findings, we attempted to apply the present method to the determination of ABPC and its metabolites in serum and urine. Figs. 4 and 5 show the separation of ABPC, and ABPC and its metabolites, from the background components of serum and urine, respectively; part A, detection at 230 nm without a reactor; B, detection at 260 nm with the HFMRs. ABPC and its metabolites were 1.5- to 3.5-times more sensitively detected at 260 nm following the postcolumn reaction, compared with direct detection at 230 nm. At an ABPC concentration of 2 μg/ml in serum samples, the R.S.D. was 4.45% ($n=15$); and at concentrations of ABPC, 1, 2 and 3 of 5 μg/ml in urine samples, the R.S.D.s were 3.89, 5.14, 5.02 and 5.55% ($n=15$), respectively. The calibration graphs of peak height *versus* concentra-

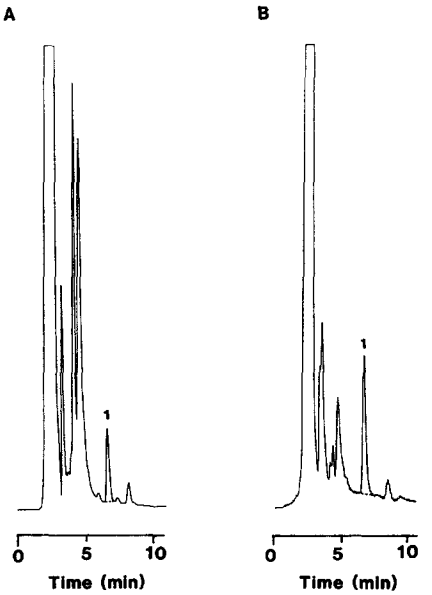

Fig. 4. Separation of ABPC from the background components of serum. A 20-μl portion of the ultrafiltrate of the serum sample was loaded onto the column. (A) Detection at 230 nm without a reactor; (B) detection at 260 nm with aminated and sulphonated HFMRs. Peak 1 is ABPC. Concentration: 5.0 μg/ml. Sensitivity: 0.032 a.u.f.s. The dotted line indicates the serum blank. Other conditions were given in the text.

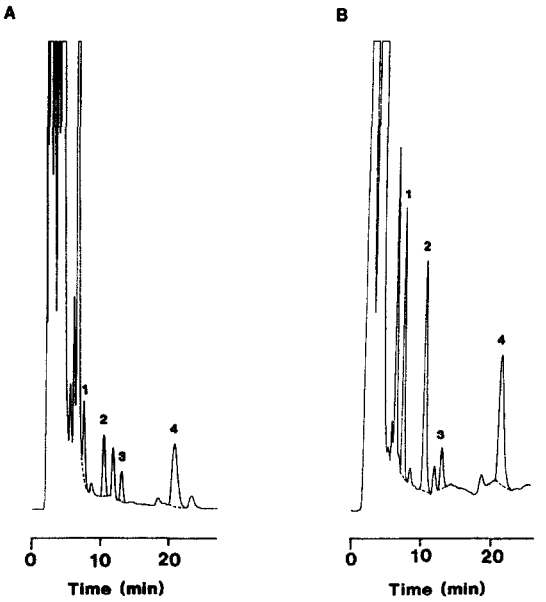

Fig. 5. Separation of ABPC and its metabolites from the background components of urine. A 20-μl portion of the filtrate of the urine sample was loaded onto the column. (A) Detection at 230 nm without a reactor; (B) detection at 260 nm with aminated and sulphonated HFMRs. Peak assignments: 1 = compound 1; 2 = 2; 3 = 3; 4 = ABPC. Concentrations: ABPC, 1 and 2, 20 μg/ml; 3, 10 μg/ml. Sensitivity: 0.032 a.u.f.s. The dotted line indicates the urine blank. Other conditions were given in the text.

tion for ABPC and its metabolites in serum and urine were linear in the concentration range 0.5–50 μg/ml with a correlation coefficient of $\geqslant 0.999$, and passed through the origin. The detection limits were 5 ng for ABPC, 1 and 2, and 20 ng for 3 at a signal-to-noise ratio of 3.

The method proposed is applicable to pharmacokinetic studies of ABPC and its metabolites after therapeutic dosage, and to their determination in bile, cerebrospinal fluid and tissues, upon slight modification.

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