Use of High Performance Liquid Chromatography for Increased Assay Sensitivity of β-Lactamase Activity in Bile

Sachiyo KUNIEDA,[a] Toshiaki NISHIHATA,*,[a] Hideo KASHIWAGI,[b] and Hiroshi TANIMURA[b]

Pharmacy Research, Upjohn Tsukuba Research Laboratories,[a] 23 Wadai, Tsukuba, Ibaraki 300-42, Japan, and Gastroenterological Surgery, Wakayama Medical College,[b] 27 Nanaban-cho, Wakayama, Wakayama 640, Japan.
Received August 18, 1992

A study to develop a sensitive method for measuring β-lactamase activity in bile was conducted. Since separation of substrate from biological components is required to increase the assay sensitivity and to achieve an accurate assay of β-lactamase activity, high performance liquid chromatography (HPLC) was used for separation and analysis of the substrates (cephaloridine for cephalosporinase, benzylpenicillin for penicillinase and cefuloxime for cefuloximase). In addition, conditions for increased assay sensitivity were also studied and optimal substrate concentrations and reaction times were determined. β-Lactamase activity of 0.05 munit/ml in bile was detected using the HPLC assay method which is a significant improvement when compared to the direct spectrophotometric method which has a detection limit of approximately 10 munit/ml.

Keywords β-lactamase; bile; HPLC; cephaloridine; benzylpenicillin; cefuroxime

β-Lactam antibiotics are widely used in the therapy of bacterial infections. However, strains of β-lactam resistant bacteria exist which are resistant to β-lactam antibiotic therapy.[1-3] One mechanism for β-lactam resistance is the inactivation of β-lactam antibiotics by β-lactamase which is produced by some strain of β-lactam resistant bacteria.[3-9]

The choice of β-lactam antibiotics for use in the therapy of bacteria infections may be aided by analysis of biological fluid for the presence of β-lactamase. Analysis of bile in the case of cholecystitis, as presented in this paper, would be one example of the usefulness of the assay. The quantitative assay of β-lactamase activity in biological samples by spectrophotometric methods is technically difficult for the following reason: [1] the presence of organic materials which interfere with the spectrophotometric quantitative analysis of substrates and [2] low activity of β-lactamase in biological samples, including the low population of bacteria, are difficult to detect by direct spectrophotometric methods.

Substrates for the β-lactamase assay by the direct spectrophotometric method, cephaloridine for cephalosporinase, benzylpenicillin for penicillinase and cefuloxime for cefuloximase should be more than a 25 μM final concentration in the assay medium.[10] When the activity of β-lactamase is low in the sample to be assayed, a decrease in substrate concentration as well as a prolongation of the reaction time may be required. The use of high performance liquid chromatography (HPLC) should provide a more sensitive method for the quantitative analysis of substrates at concentrations of less than 25 μM.

In the present study, a highly sensitive assay method for detection of β-lactamase activity in bile from a patient with cholecystitis was demonstrated by using high performance liquid chromatography (HPLC).

Materials and Methods

Materials Cephaloridine (CER), benzylpenicillin (PG), cefuloxime (CFX) and standard β-lactamase were obtained commercially from Sigma Chemicals (Mo, U.S.A.). Other reagents used were of analytical grade.

Assay of β-Lactamase Activity The accuracy and precision of the assay method was determined by using phosphate buffer (pH 7.4) containing a designated concentration of either CER, PG or CFX as substrate (as substrate solution) and human bile without β-lactamase activity which was diluted with the phosphate buffer containing 2.0% Triton X-100 (1 : 1) (as β-lactamase solution). Either 0.5 or 1.0 ml of the β-lactamase solution was added to 4.0 ml of the substrate solution at 30 °C. Two hundred and fifty μl samples were collected at designated time intervals and 750 μl of acetonitrile (containing β-naphtylacetic acid as an internal standard for the HPLC assay) was added to the samples to stop the enzyme reaction. The samples were centrifuged at 900 g for 10 min. After collection, the supernatant was dried and the residue was dissolved in 500 μl of mobile phase for the HPLC assay.

Assay of β-lactamase in bile samples collected from 3 patients with cholecystitis which contained bacteria with β-lactamase was performed as follows: 1 ml of bile was mixed with 1 ml of the phosphate buffer containing 2.0% triton X-100 to prepare the assay sample as β-lactamase solution. The assay procedure performed is described above.

HPLC of Substrates CER, PG and CFX were analyzed by HPLC under the following conditions; Wako-pak Neuclosil 5C18 (reverse phase column material); and a column length of 25 cm (4.6 mm, i.d.). The mobile phase for analysis of substrates consisted of acetonitrile and 0.05 M phosphate buffer at pH 6.5 (15 : 85) and the flow rate was 1.2 ml/min (pressure of about 100 kg/cm^2). A spectrophotometric detection at 254 nm was for CER and CFX, and at 220 nm for PG. Typical chromatogram of CER, PG and CFX are shown in Fig. 1.

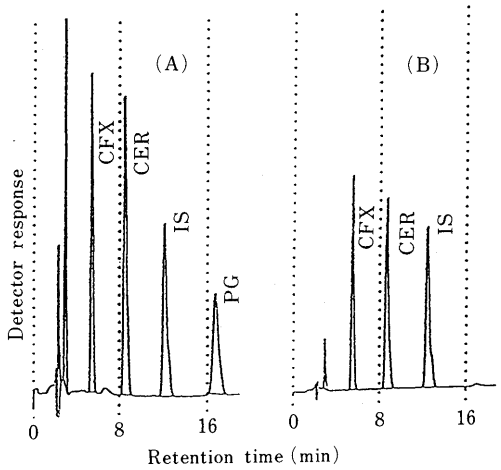

Fig. 1. Typical Chromatogram of Substrates, CER (2.5 μM), PG (2.5 μM) and CFX (2.5 μM) and Internal Standard (0.6125 μg/ml) after Extraction from Bile

A, detected at 220 nm and B, detected at 254 nm.

Results and Discussion

Calibration Curve for CER, PG and CFX by HPLC Calibration curves for CER, PG and CFX are shown in Fig. 2. The ratio of the peak area of each substrate against that of the internal standard is plotted against the concentration of each CER, PG, and CFX in the medium. CER and CFX at a concentration of less than 0.4 μM and PG at that of less than 2.5 μM was not analyzed quantitatively in the present study (analytical limitation). The molecular extinction coefficient of CER and CFX is about 15000 at 254 nm and that of PG is about 5000 at 220 nm. This difference accounts for the difference in the analytical limitation as well as to the delayed retention time for PG.

Assay of Standard β-Lactamase Activity Investigation of the assay conditions for the measurement of β-lactamase activity in bile samples was performed with standard β-lactamase. Since the minimum analytical concentration of CER, CFX and PG was at concentrations of 0.40 or 2.5 μM in the medium, a concentration of 2.5 or 10 μM of substrate was used as the minimum substrate concentration in the medium for assay of β-lactamase.

The β-lactamase activity for the substrate CER was examined at calculated activities of 0.05 to approximately 500 munit/ml. The assay conditions used are shown in Table I. As indicated in Fig. 3, it was observed that degradation of CER by the enzyme occurred with apparent zero-order kinetics; *i.e.*, the substrate concentration used for each β-lactamase solution satisfied the condition of $[S] \gg K$ ($[S]$ = substrate concentration; K = equilibrium constant of complex formation of substrate and enzyme). When the concentration of 5 μM of CER was used for the assay of β-lactamase at 550 munit/ml, the reaction did not satisfy the condition to achieve apparent zero-order kinetics of degradation (Fig. 3B). Further, when the activity of β-lactamase was very low (0.05 munit/ml), prolongation of the reaction time allowed assay of β-lactamase activity (Fig. 3F). Thus, the substrate concentration and reaction time conditions for the precise assay of β-lactamase activity in bile samples were established. The activity measured as cephalosporinase against CER which was obtained experimentally was consistent with the calculated activity for the standard as shown in Table I.

In regards to the β-lactamase activity of the standard using PG as the substrate, the β-lactamase activity measured as penicillinase which was obtained experimentally was consistent with the calculated activity of the standard which is illustrated in Table I. Although the standard β-lactamase degraded CFX, it was not clear whether a complete and accurate measurement of the activity was achieved in the assay because there was no information concerning the

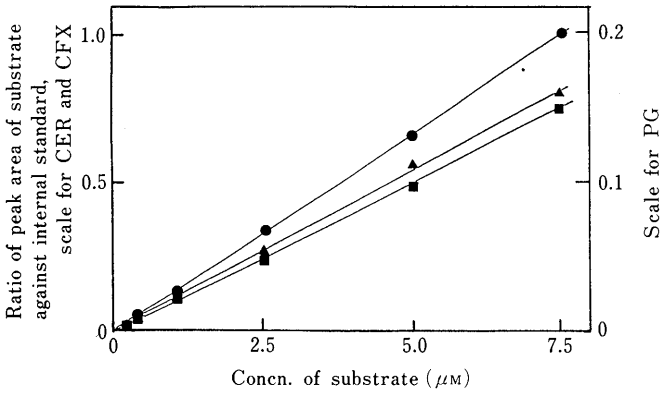

Fig. 2. Calibration Curves of CER (●), PG (▲) and CFX (■)

$Y=0.13X+0.0011$ ($r=0.999$) for CER; $Y=0.021X+0.0020$ ($r=0.998$) for PG; $Y=0.10X-0.0027$ ($r=0.999$) for CFX.

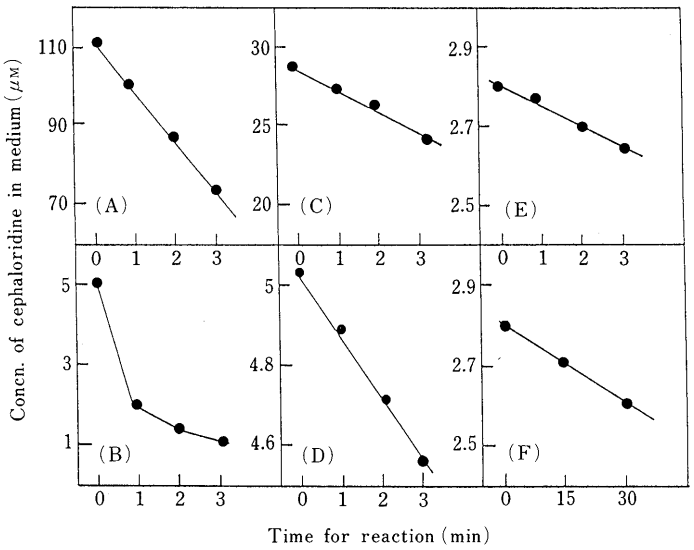

Fig. 3. Profiles of Degradation of CER in the Reaction Medium by Standard β-Lactamase under the Condition Described in Table I

Concentration of β-lactamase and CER, respectively; (A) 550 munit/ml and 100 μM; (B) 550 munit/ml and 5 μM; (C) 27.5 munit/ml and 25 μM; (D) 5 munit/ml and 5 μM; (E) 0.5 munit/ml and 2.5 μM; and (F) 0.05 munit/ml and 2.5 μM.

TABLE I. Assay Conditions Tested Using a Standard β-Lactamase in the Medium Containing Human Bile

Exp. group	Standard β-lactamase activity, calculated in sample (munit/ml)	Concn. of substrate in medium (μM)	Reaction time (min)	Measured β-lactamase activity (munit/ml)
1	550	CER 100	0, 1, 2, 3	530±30
2	27.5	CER 25	0, 1, 2, 3	28±4
3	7.2	CER 10	0, 1, 2, 3	7.1±0.8
4	5.0	CER 5 to 10	0, 1, 2, 3	5.1±0.5
5	0.5	CER 2.5 to 10	0, 1, 2, 3	0.47±0.08
6	0.05	CER 2.5 to 10	0, 10, 20, 30	0.046±0.007
7	0.72	PG 2.5 to 10	0, 10, 20, 30	0.77±0.09
8	Unknown	CFX 2.5 to 10	0, 10, 20, 30	0.19±0.03

TABLE II. β-Lactamase Activity in Bile Collected from Cholecystitis Patients

	Substrate and concn. (μM)	Reaction time (min)	β-Lactamase activity in bile (munit/ml)
Patient A	CER 10	0, 10, 20, 30	0.26
	PG 10	0, 10, 20, 30	0.22
	CFX 10	0, 10, 20, 30	0.66
Patient B	CER 10	0, 10, 20, 30	ND
	PG 10	0, 10, 20, 30	ND
	CFX 10	0, 10, 20, 30	0.8
Patient C	CER 10	0, 10, 20, 30	9.4
	PG 10	0, 10, 20, 30	0.16
	CFX 10	0, 10, 20, 30	ND

ND represents the undetected (less than 0.05 munit/ml).

activity of the standard enzyme expressed as cefuloximase.

Assay of β-Lactamase in Bile, Obtained from Cholecystitis Patients The assay of β-lactamase activity in bile obtained from 3 patients was conducted by using all 3 substrates (CER, PG and CFX). The assay conditions listed in experimental group 6 in Table I were used for the assay of the clinical specimens because of the low activity of β-lactamase in the bile samples. As shown in Table II, the bile obtained from patient A possessed cephalosporinase, penicillinase, and cefuloximase. However, the bile samples obtained from the other two patients lacked activity against at least one of the three substrates.

It was demonstrated in the present study that the use of HPLC for quantitative analysis of the substrate for β-lactamase could decrease the substrate concentration required for tthe β-lactamase assay and avoid interference by biological components with complete separation of the substrate from the biological component on the chromatogram. The HPLC method was quantitative for β-lactamase activity in bile samples at 0.05 munit/ml. Since it has been reported[10] that β-lactamase activity less than 10 munit/ml was not detected by the direct spectrophotometric method, the HPLC method in the present study resulted in increased sensitivity for assay of β-lactamase activity.

Acknowledgement The authors thank Dr. J. E. Lund of the Upjohn Tsukuba Res. Labs. for his technical assistance on the manuscript.

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