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Sensitive determination of a β-lactam antibiotic, cefaclor by liquid chromatography with chemiluminescence detection

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

We present a sensitive method for the determination of cefaclor (CCL), a β-lactam antibiotic. It was based on the chemical derivatization of the drug with 4-(2′-cyanoisoindolyl)phenylisothiocynate (CIPIC) under the reaction conditions with heating at 80 °C for 7 min in the presence of pyridine. The CIPIC reagent could react with the primary amino group of the drug to form the CIPIC-conjugated CCL. The derivatives emitted not only fluorescence (FL) at maximum emission wavelength of 410 nm with irradiation at 310 nm, but also chemiluminescence (CL) in the presence of H_2O_2, borate buffer (pH 9.6) and acetonitrile. After separation of the CIPIC derivatives of CCL and cephradine as internal standard in human serum by reversed-phase liquid chromatography, the derivatives could be monitored with both FL and CL detections. The detection limit (S/N = 3) in the chromatograph was 1 pmol by the CL detection and 10 pmol by the FL detection. The proposed CL method permitted the most sensitive determination of CCL in the human serum after its oral administration.

Keywords: Cefaclor; Chemiluminescence; Fluorescence; Derivatization; Drug monitoring; Sensitive determination

1. Introduction

Cefaclor (CCL) is a β-lactam antibiotic of the cephalosporin group, which expresses a potential activity against many bacteria infections, and thus has been widely used as an oral medicine for the infectious diseases. Several methods have been developed for the determination of β-lactam antibiotics. For example, Gupta and Prasad [1] recently reported a spectrophotometric method based on the molecular recognition of CCL by a silica gel-bound cationic polyelectrolyte, in which the electrolyte was used for solid phase extraction for the clean-up and enrichment of the analyte in blood

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and urine specimens, and finally UV-spectrometric measurement was conducted after acid conversion of CCL into its oxazolone compound. Spectrofluorometric method [2] utilizing fluorogenic reaction of primary amino group with fluorescamine was reported for the batch measurement of β-lactam antibiotics. Besides, HPLC and capillary electrophoresis methods [3–7] were also employed for the simultaneous determination of β-lactam antibiotics in biological specimens, in which UV detection was generally performed. However, the sensitivity of those methods was fairly low and the detection limit was 100 pmol level. A highly sensitive and quantitative method still would be desirable to reduce the sample volume.

We studied a chemiluminometric and fluorometric detection method for the sensitive determination of CCL in blood serum. As shown in Fig. 1, CCL and cephradine as internal standard were first reacted with our developed fluorescent Edman-type reagent, 4-(2′-cyanoisoindolyl)phenylisothiocynate (CIPIC) [8], and the formed derivatives in the reaction mixture were separated by reversed-phase liquid chromatography. After the separation, the CIPIC derivatives were detected with a fluorescence (FL) detector, and then successively oxidized with H_2O_2 in an alkaline borate solution containing acetonitrile to sensitively detect the chemiluminescence (CL) intensities from the derivatives.

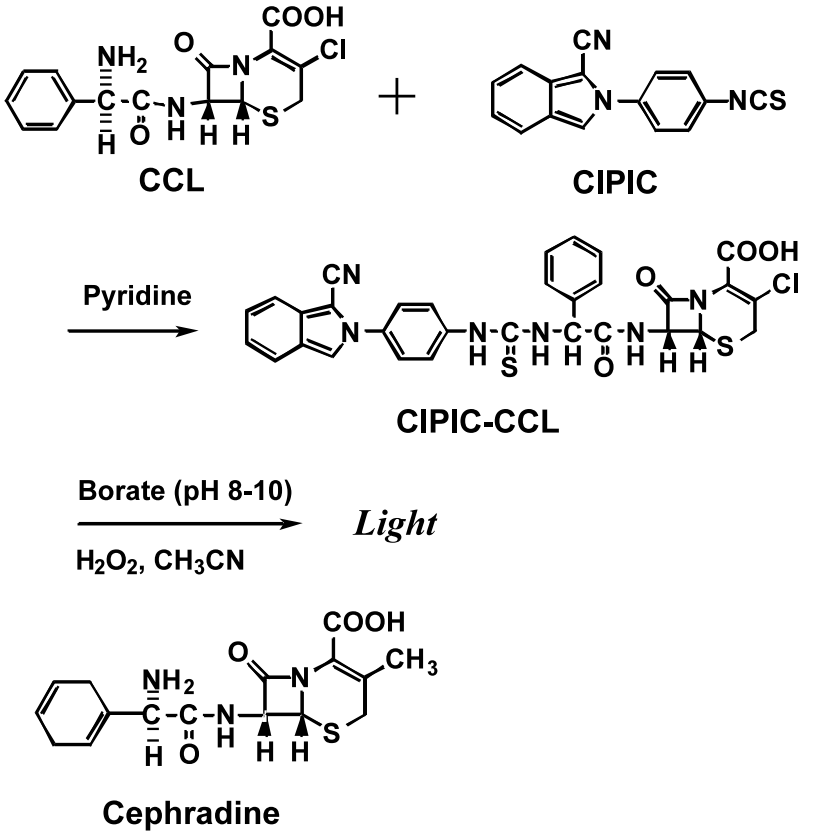

Fig. 1. Derivatization scheme for the reaction of CCL with CIPIC and its CL reaction, and the chemical structure of cephradine used for internal standard.

2. Experimental

2.1. Reagents and solutions

CCL and cephradine were products of Sigma Chemical Co. (St. Louis, MO), and CCL capsules (500 mg each) were obtained from Shionogi Pharmaceutical Co. (Osaka, Japan). CIPIC was synthesized according to the literature [8], and dissolved in acetonitrile for appropriate concentrations. All other chemicals were of analytical-reagent grade and were used as received. Pure water was prepared using MILLI-XQ equipment. Stock solutions (10 mM) for CCL and cephradine were prepared by dissolving in water, and serial dilutions were conducted with same solvent. Hydrogen peroxide solution (1.5 M) used for the CL reaction was prepared by dilution of 15 ml of 30% H_2O_2 aqueous commercial solution with 85 ml of acetonitrile. Blood sera were obtained from a healthy female volunteer (23 years old).

2.2. Typical procedure for the derivatization with CIPIC

To 40 μl of serum, were added 10 μl of CCL (0–100 μM) and/or 20 μM cephradine as internal standard, 25 μl of pyridine and 50 μl of 35 mM CIPIC. The mixture was reacted at 80 °C for 7 min in a heater, and then cooled in ice water. The mixture was centrifuged at approximately 1000 × *g* for 15 min. The supernatant (25 μl) containing the CIPIC-conjugated CCL (CIPIC–CCL) derivative was used for HPLC after filtration with a 0.45-μm pore size filter.

2.3. Apparatus

FL spectra were measured manually with a Hitachi (Tokyo, Japan) 650–10 spectrometer using a 1.0-ml semimicro-quartz cell. Time-dependent CL intensity was measured manually with a photon-counting computer-controlled BLR-201 Aloka luminometer (Tokyo, Japan) using 75 × 12 mm^2 round-bottom glass tubes.

2.4. Chromatographic conditions and its optimum operation

The HPLC system consisted of a PU-980 pump (Jasco, Tokyo, Japan), a LG-980-02 gradient unit (Jasco) equipped by a low pressure degasser, a 7125 injector (Rheodyne, Cotati, CA), a FP-920 FL detector (Jasco) and a 825-LC CL detector (Jasco).

For the quantitative analysis of CCL, the CIPIC–CCL derivative in the reaction mixture was separated on a TSKgel ODS-80TM reversed-phase column (150 × 4.6 mm^2 i.d., 5-μm particle size, Tosoh, Japan) by isocratic elution of 35% (v/v) acetonitrile in mobile phase and 10% (v/v) of 0.1 M triethylamine (pH 8.5) adjusted the pH with 0.1 M acetic acid. The flow rate of the mobile phase was 0.6 ml/min. The fluorescent peaks were monitored at wavelengths of 310 nm for excitation and 410 nm for emission.

As shown in Fig. 2, the column eluate after the FL detection was introduced to on-line post-column reaction system for the CL detection. In the system, a mixture of 0.1 M sodium borate buffer (pH 9.6) and acetonitrile (3:2, v/v), and 1.5 M H_2O_2 in acetonitrile and H_2O (17:3, v/v) were added to the eluate stream with two reagent-delivery pumps at each flow-rate of 0.2 ml/min. The mixture was passed through a stainless coil (2.2 m × 0.5 mm i.d.) located prior to the CL detector. The CL intensity in the final eluate was then monitored.

3. Results and discussion

3.1. Optimization of the derivatization conditions

The optimum reaction conditions for the production of the CIPIC–CCL were investigated using reversed-phase HPLC with FL detection. CCL (25 μM) in serum (40 μl) could react well with CIPIC in the presence of a basic catalyst of 20% (v/v) pyridine in the reaction mixture; in the absence, the production was reduced to approximately half (Fig. 3A). To obtain maximum production of CIPIC–CCL in serum (Fig. 3B), the optimum concentration of CIPIC was 14 mM in

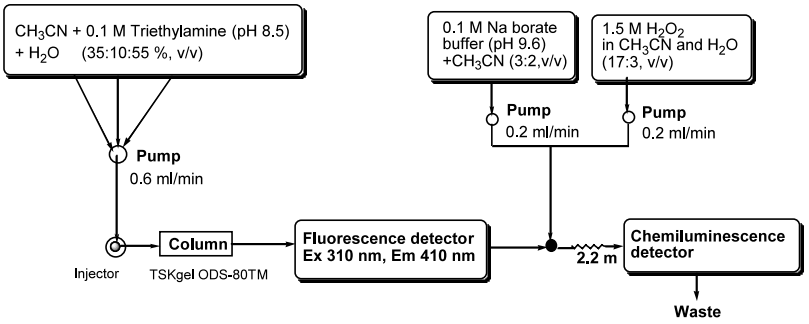

Fig. 2. HPLC system with FL and CL detections for the determination of CCL derivatized with CIPIC.

the reaction mixture; 35 mM CIPIC solution was used. When the authentic CCL only was used for the derivatization reaction, 5 mM CIPIC solution gave the same production as that obtained with 35 mM CIPIC for CCL in serum. This indicated that a higher concentration of CIPIC needed for the production of CIPIC–CCL in serum, because many other amino substances in serum consumed CIPIC. The reaction time (0–15 min) at 80 °C was investigated; the production reached maximum between 5 and 10 min (Fig. 3C). Thermostatting at 80 °C for 7 min was selected for reproducible results. During the reaction, proteins in the reaction mixture were denaturated. Thus the denaturated proteins were separated by centrifugation and filtration before HPLC analysis.

3.2. HPLC separation and detection

The CIPIC–CCL derivative in the reaction mixture was separated from other interfering compounds on a reversed-phase HPLC column (TSK gel ODS-80TM) by isocratic elution of the mobile phase containing 35% acetonitrile and 10% 0.1 M triethylamine (pH 8.5). CIPIC and its conjugated amino acid derivatives were found to emit CL by the oxidation reaction with alkaline borate buffer, H_2O_2 and acetonitrile [9]. After the FL detection, the column eluate was thus successively mixed with sodium borate buffer (pH 9.6) and H_2O_2 solution containing acetonitrile in order to detect the CL intensity, as shown in Fig. 2. The CIPIC–CCL derivative was eluted at 31 min, and

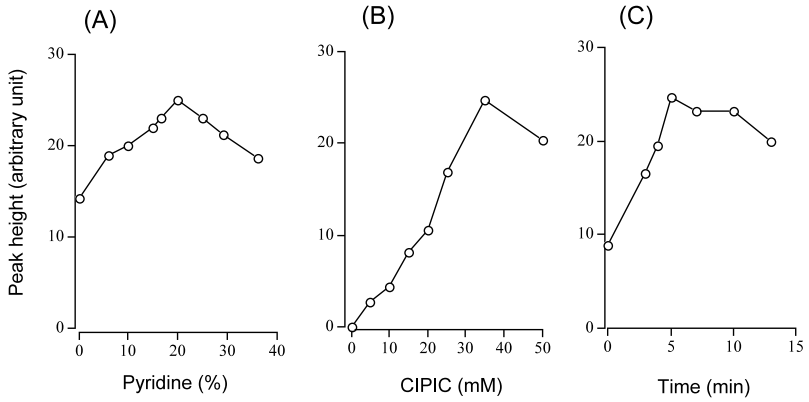

Fig. 3. Effects of concentrations of pyridine (A) and CIPIC (B), and reaction time (C) on the derivatization of CCL in serum with CIPIC.

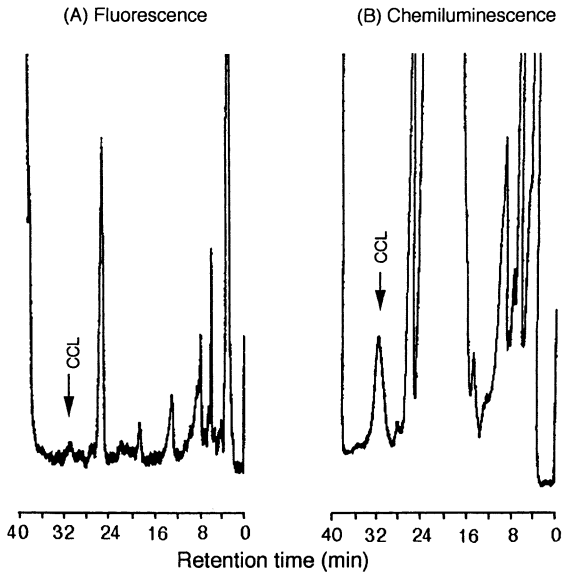

Fig. 4. FL (A) and CL (B) detections in HPLC of a reaction mixture of CCL with CIPIC. 1.0 μM CCL (50 μl) was used for the derivatization reaction, and its 10 pmol amount was injected to the chromatograph.

at 40 min excess CIPIC was eluted (Fig. 4). The FL peaks of the compounds were detected at maximum emission and excitation wavelengths of 410 and 310 nm, respectively. The FL peak of CIPIC–CCL in the chromatogram corresponded to its CL peak. The sensitivity of the FL detection was approximately ten times lower than that of the CL detection. The lower detection limit of the CCL–CIPIC compound was 1 pmol by the proposed CL detection system that was optimized by following experiments.

The CL intensity of the CIPIC–CCL peak was influenced by the length of the mixing coil located prior to CL detector (Fig. 5A), since the intensity varied with pH of the borate buffer used for the CL reaction, and a maximum intensity at pH 9.6 was obtained at approximately 90 s after initiating the reaction (Fig. 6). In this HPLC detection system, the column eluate was passed through a stainless coil (2.2 m × 0.5 mm i.d.), not only for adjusting the reaction time but also for mixing the oxidant reagents before the CL detection. As shown in Fig. 5B, the CL intensity increased with increasing concentration of the borate buffer (pH 9.6). Finally 0.1 M borate buffer was selected, since the buffer with a concentration higher than 0.15 M was not soluble in the final reaction mixture. As shown in Fig. 5C, 1.0–2.5 M H_2O_2 gave a maximum and constant CL intensity and thus 1.5 M H_2O_2 was employed for the system.

In this post-column reaction system, the final eluate contained approximately 46% (v/v) acetonitrile. The CL intensity from CIPIC–CCL resulted in approximately sixfold increase as compared with that obtained without acetonitrile in the post-column reagent solutions of borate buffer and H_2O_2.

3.3. Drug monitoring

Human sera were only deproteinized by denaturation. This denaturation was conducted by

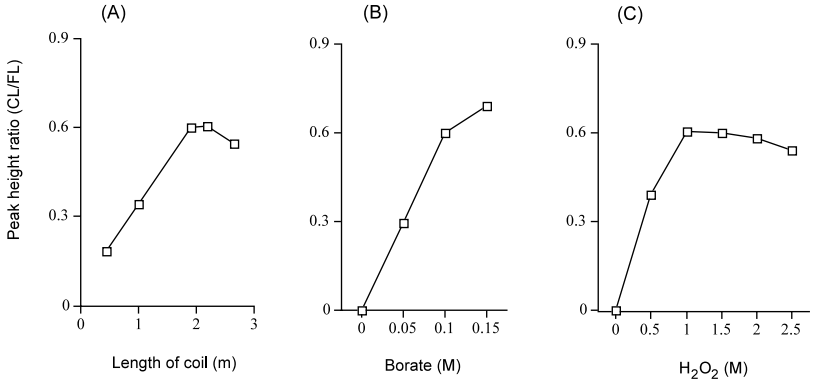

Fig. 5. Effect of length of the CL reaction coil (A) and concentrations of pH 9.6 borate buffer (B) and H_2O_2 (C).

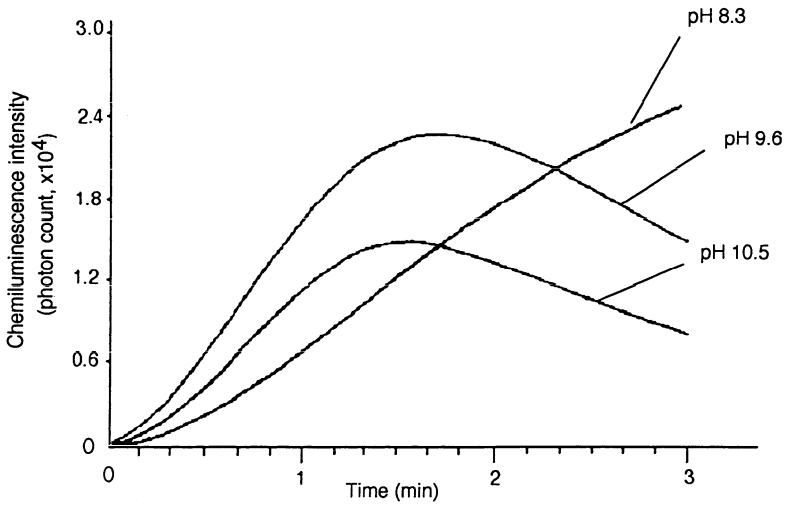

Fig. 6. Time-course of the CL development from 33 μM CIPIC after mixing with 17 mM borate buffer (pH 8.3–10.5), 0.17 M H_2O_2 and 33% CH_3CN.

heating the samples during the derivatization reaction. Thus, this pretreatment for the biological samples was very simple. Fig. 7 shows chromatograms of human serum dosed with CCL, that was obtained by the proposed HPLC system with the FL and CL detections. In this assay, a cephalosporin drug, cephradine was used as internal standard, since its chemical structure (Fig. 1) was very similar to that of CCL.

The within-day reproducibility of the CL and FL intensity and their peak height ratio to the internal standard was evaluated from the results of five repeated measurements of CCL in serum (Table 1). The relative standard deviation (RSD)

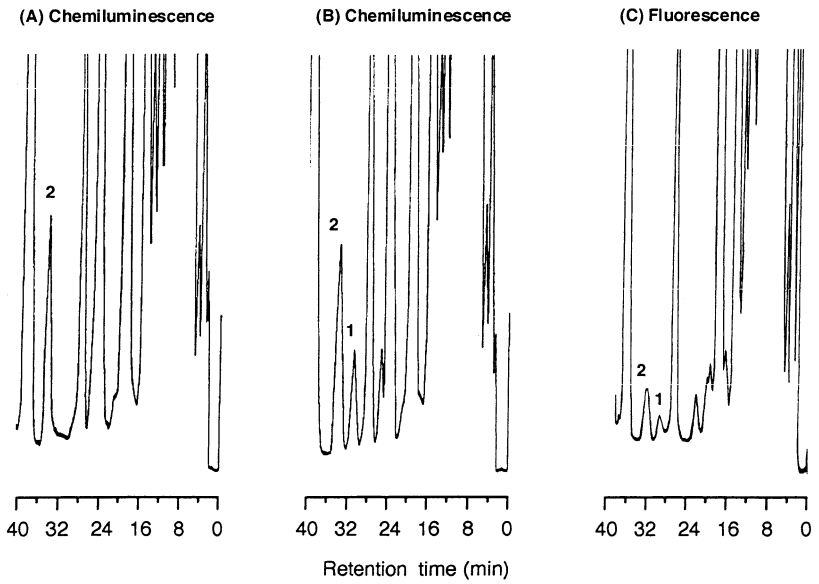

Fig. 7. Chromatograms obtained by CL and FL detections of serum at 3 h after oral administration of CCL, and its sample was spiked without (A) or with (B and C) cephradine as internal standard. Peaks: (1) 5 μM internal standard, (2) 10.6 μM CCL in serum.

Table 1
Evaluation of assay repeatability for 10 μM CCL using 5 μM cephradine as internal standard

	CL		FL	
	Peak height	Ratio	Peak height	Ratio
	78	2.58	42	2.40
	129	2.15	58	2.76
	94	2.19	48	2.40
	81	2.44	61	2.42
	89	2.41	52	2.78
RSD (%)	19.5	6.9	13.1	7.0

was 6.9–7.0% for the ratio and 19.5–13.1% for the peak height. The results indicated that the internal standard method was more precise. The variation of the peak height might be caused by the varied production yield of the CIPIC–CCL and/or by the error of its injected volume into the chromatogram. Therefore, the calibration graph was made by plotting the ratio of the peak height against the concentration of CCL in serum (0, 5, 10, 20, 25 μM, $n = 2$ for each). The regression equation for the graph was $Y = 0.282\ X - 0.162$ by the FL detection, and $Y = 0.261\ X + 0.229$ by the CL detection, in which Y and X represent the ratio and the concentration, respectively. Each correlation coefficient (r^2) for the straight line was 0.986 and 0.992, respectively. The concentration-time graph for CCL in serum after administration of CCL is shown in Fig. 8. The measurements were performed by the CL detection, since the FL detection was not sufficient for the determination of the CCL concentration lower than 1 μM. Its half-life in serum after the administration was 1.04 h. There were no data in other reports for the measurements of CCL in human blood after dosed with the oral administration.

In conclusion, the present HPLC method coupled with FL and CL detections can be applied to the quantitative determination of CCL in human serum. The proposed protocol permits the assay of the CCL serum concentrations higher than 1 μM in serum by the FL detection, and 0.1 μM by the CL detection. This study also provides first CL method for the determination of CCL. The CL method requires a simple procedure for the serum treatment, and its sensitivity is the highest compared with previously reported methods.

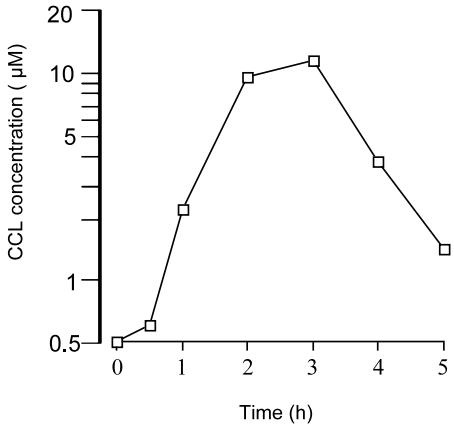

Fig. 8. Time-concentration curve of CCL in serum after oral administration (500 mg dose).

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