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Talanta 60 (2003) 325–334
Talanta
www.elsevier.com/locate/talanta

Chromatographic determinations of a β-lactam antibiotic, cefaclor by means of fluorescence, chemiluminescence and mass spectrometry

Masaaki Kai*, Hiromi Kinoshita, Mikio Morizono

School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-Machi 1-14, Nagasaki 852-8521, Japan

Received 17 June 2002; received in revised form 7 October 2002; accepted 16 October 2002

Abstract

We report analytical informations on the quantification of cefaclor (CCL), a β-lactam antibiotic by three detection methods. The methods were based on the chemical derivatization of the drug with 4-(2′-cyanoisoindolyl)phenylisothiocyanate (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 CIPIC-conjugated CCL. The derivative emitted not only fluorescence (FL) at maximum emission wavelength of 410 nm with the irradiation at 310 nm excitation, but also chemiluminescence (CL) in the presence of H_2O_2, borate buffer (pH 9.5) and acetonitrile. After reversed-phase liquid chromatographic separation of the CIPIC-conjugated CCL in blood, the derivative could be monitored with a FL detector, indicating the detection limit (S/N = 3) of 10 pmol/injection. The CIPIC-conjugated CCL was further monitored most sensitively by a CL detector after simply mixing H_2O_2 and borate buffer with the column eluate. The CL detection limit was 1.0 pmol/injection. In addition, we attempted to detect the CIPIC-conjugated CCL by liquid chromatographic-mass spectrometry (LC/MS). The MS method permitted the specific detection of the CIPIC derivative of the drug, though the sensitivity was 10^4-times lower than that of the CL detection.

Keywords: Cefaclor; Chemiluminescent determination; Fluorescent determination; Mass spectrometry

1. Introduction

Cefaclor (CCL) is a β-lactam antibiotic of the cephalosporin group, which express a potential activity against many bacteria infections, and thus has been widely used as an oral medicine for infectious diseases. Several methods have been developed for the determination of β-lactam antibiotics. For example, a spectrophotometric method using an immobilized polyeletrolyte was recently reported for the determination of CCL in blood and urine, in which the polyeletrolyte was used for clean-up and enrichment of the analyte, and finally UV detection was conducted after acid conversion into its oxazolone [1]. Spectrofluorometric method utilizing the fluorogenic reaction of a primary amino group with fluorescamine was

* Corresponding author. Tel./fax: +81-95-843-1742.
E-mail address: ms-kai@net.nagasaki-u.ac.jp (M. Kai).

0039-9140/03/$ - see front matter
doi:10.1016/S0039-9140(03)00069-9

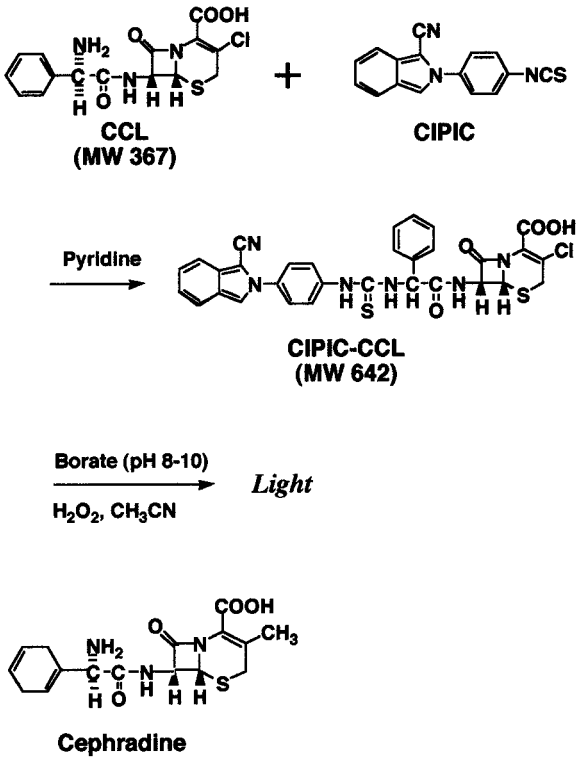

Fig. 1. Derivatization scheme for the reaction of CCL with CIPIC and its CL reaction, and chemical structure of cephradine used for internal standard.

reported for the batch measurement of β-lactam antibiotics [2]. In addition, HPLC [3–7] and capillary electrophoresis [8] methods have been studied 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 ca. 100 pmol level. A highly sensitive and quantitative method still would be desirable to reduce the sample volume.

In this report, we studied three methods based on fluorometric, chemiluminometric and mass spectrometric detections for the sensitive determination of CCL. In the methods, CCL in human serum and cephradine as internal standard were first reacted with our developed fluorescent Edman-type reagent, 4-(2′-cyanoisoindolyl)phenylisothiocyanate (CIPIC) [9] to form the CIPIC-conjugated CCL (Fig. 1). The CIPIC derivatives after reversed-phase chromatographic separation were detected with a fluorometer, and successively oxidized with H_2O_2 in alkaline borate buffer containing acetonitrile to detect the chemiluminescence (CL) intensities from the derivatives (Fig. 2). The CIPIC-conjugated CCL (MW = 642) and unmodified CCL (MW = 367) were detected by LC/MS with atmospheric pressure chemical ionization (APCI).

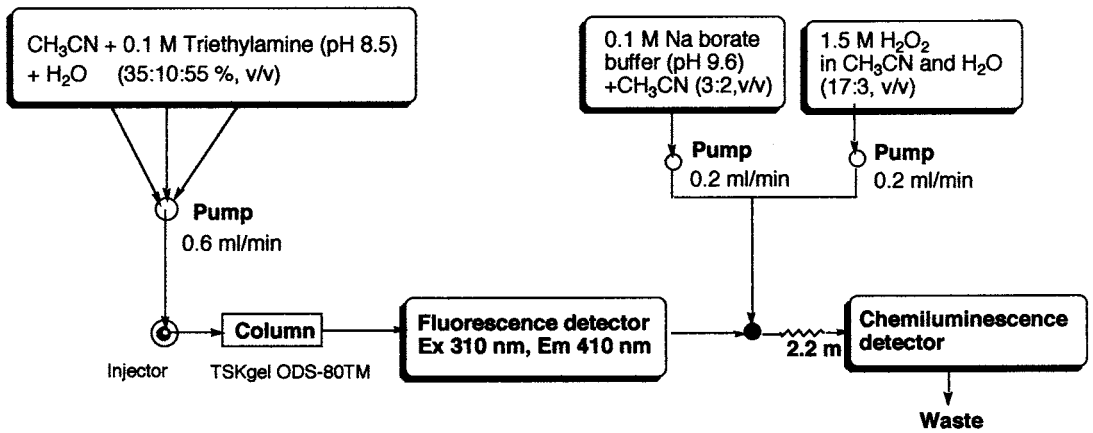

Fig. 2. HPLC system with FL and CL detections for the determination of CCL derivatized with CIPIC.

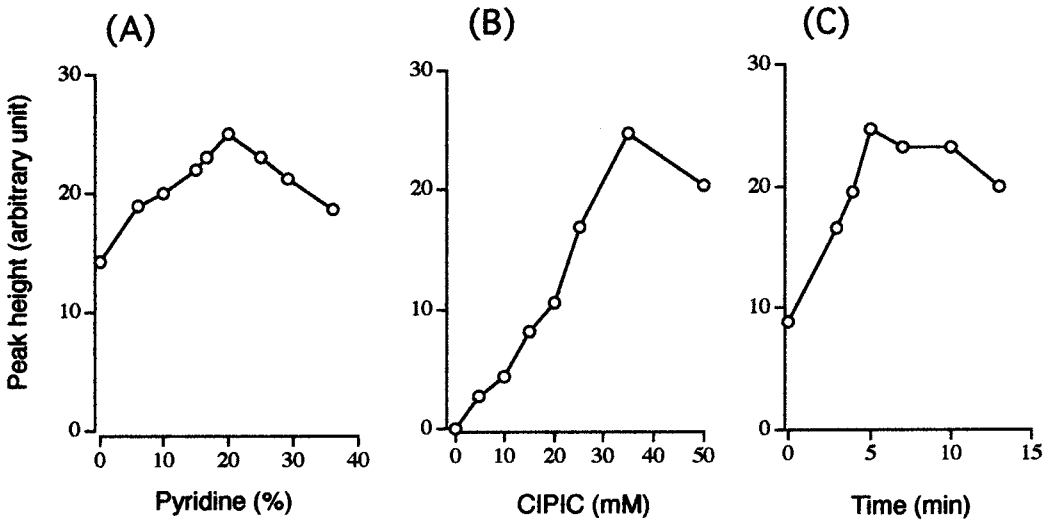

Fig. 3. Effects of concentrations of pyridine (A) and CIPIC (B), and reaction time (C) on the derivatization of CCL in serum with CIPIC.

2. Experimental

2.1. Reagents and solutions

CCL and cephradine were products of Sigma Chemical Co. (St. Louis, MO, USA), and CCL capsules (500 mg each) were obtained from Shionogi Pharmaceutical Co. (Osaka, Japan). CIPIC was synthesized according to the literature [9], 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) required for the CL reaction was prepared by dilution of 15 ml of 30% H_2O_2 aqueous solution with 85 ml of acetonitrile. Blood serum was obtained from a healthy female volunteer (23 years old), and kept frozen at -80 °C for the use of the experiment of the reaction conditions. The administered blood samples were kept at 4 °C, and used within a day.

2.2. Typical procedure for reaction 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 ca. $1000 \times g$ for 15 min. The supernatant (25 μl) containing the CIPIC-CCL derivative was used for HPLC after filtration with a 0.45-μm pore size filter.

2.3. Manual measurement

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 round-bottom glass tubes.

2.4. HPLC apparatus and its operation

The HPLC system consisted of a PU-980 pump (JASCO, Tokyo, Japan), a LG-980-02 gradient

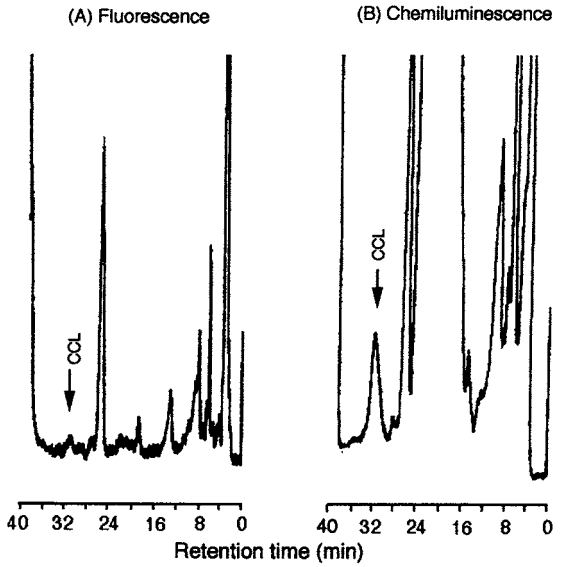

Fig. 4. FL (A) and CL (B) detections in HPLC of a reaction mixture of CCL with CIPIC. One micromolar CCL (50 μl) was used for the derivatization reaction, and its 10 pmol amount was injected to the chromatograph.

unit (JASCO, Tokyo, Japan) equipped by a low pressure degasser, a 7125 injector (Rheodyne, Cotati, CA, USA), a FP-920 FL detector (JASCO, Tokyo, Japan) and a 825-LC CL detector (JASCO, Tokyo, Japan).

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 i.d., 5-μm particle size, Tosoh, Japan) by isocratic elution of 34% (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^{-1}. 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^{-1}. 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.

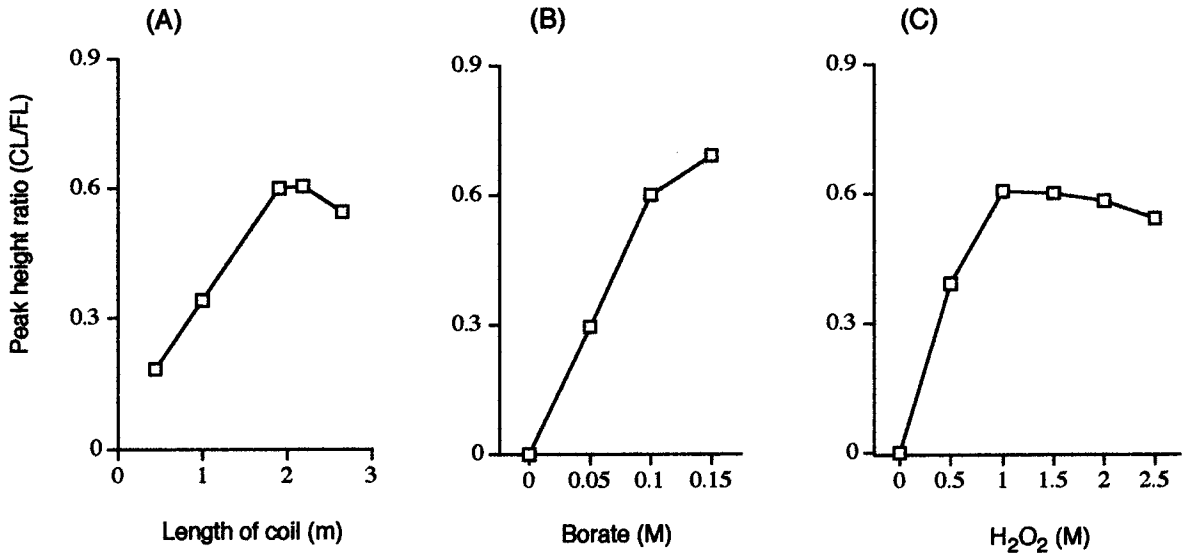

Fig. 5. Effect of length of the CL reaction coil (A) and concentrations of pH 9.6 borate buffer (B) and H_2O_2 (C).

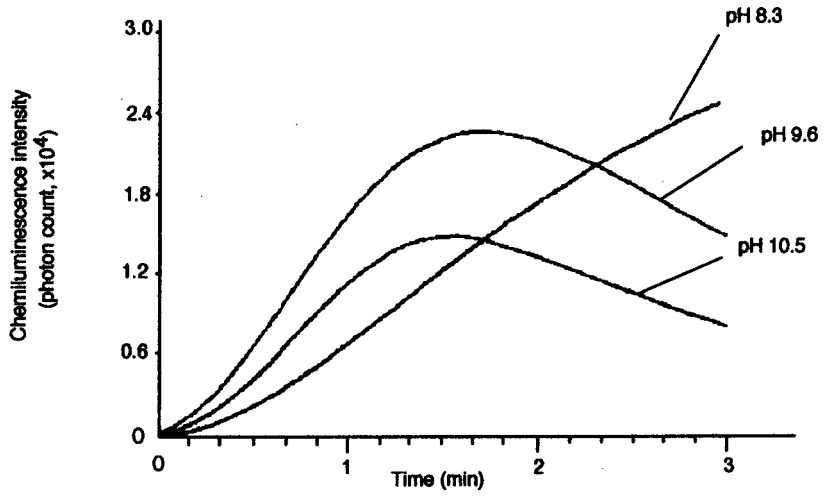

Fig. 6. Time-course of the CL development from 33 μM CIPIC after mixing with 17 mM borate buffer (pH 8.3–10.5), 0.17 M H_2O_2 and 33% CH_3CN.

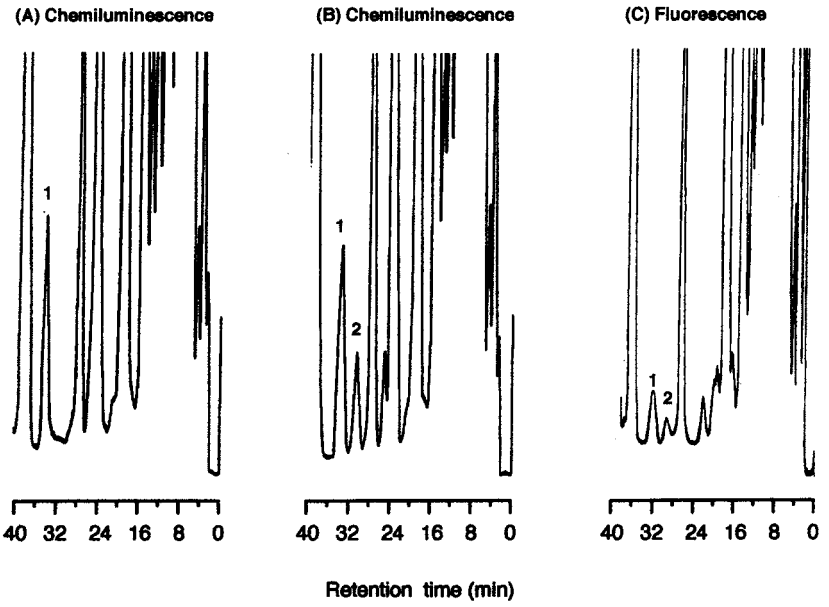

Fig. 7. Chromatograms obtained by CL (A and B) and FL (C) detections of serum at 3 h after oral administration of CCL, and its sample spiked with cephradine as internal standard. Peaks: 1, 5 μM internal standard; 2, 10.6 μM CCL in serum.

2.5. LC/MS operation

For the APCI-MS detection of CIPIC-conjugated CCL, a portion (50 μl) of the derivatization reaction mixture was injected to the above reversed-phase HPLC column and then separated by isocratic elution with the mobile phase composed of 40% (v/v) acetonitrile and 10% (v/v) 0.1 M triethylamine acetate (pH 8.5). The column effluent was introduced into an M1000 mass spectrometer (Hitachi, Tokyo, Japan) equipped with APCI interface. The nebulizer and vaporizer

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

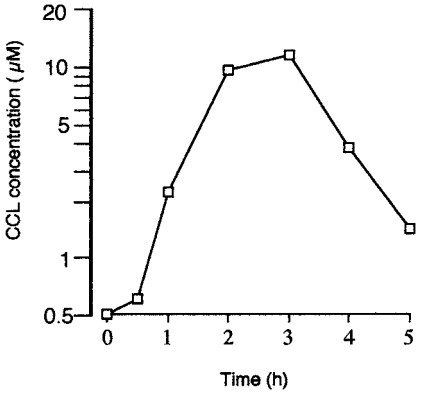

Fig. 8. Time–concentration curve of CCL in serum after oral administration (500 mg dose).

temperatures of the APCI interface were set to 230 and 390 °C, respectively. Mass spectra were obtained in the cyclic scan mode capable of scanning *m/z* 100–900 positive ions at 4 s per scan. For the quantitative measurement, selected-ion monitoring (SIM) was performed, in which the quasi-molecular ions of CCL and CCL–CIPIC were selectively monitored.

3. Results and discussion

3.1. Optimization of the derivatization conditions

The optimum reaction conditions for the production of the CIPIC-conjugated CCL (CIPIC-CCL) were investigated using reversed-phase HPLC with FL detection. CCL (25 μM) in serum 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 ca. half (Fig. 3A). To obtain maximum production of CIPIC-CCL in serum (Fig. 3B), the optimum concentration of CIPIC was 14 mM in 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). Thermosetting 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. The reaction conditions may slightly decompose CCL molecules, however this derivatization technique allows the sensitive quantification as demonstrate below.

3.2. FL and CL detections

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 [10]. 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 at 40 min excess CIPIC was eluted. The FL peaks of the compounds were detected at maximum

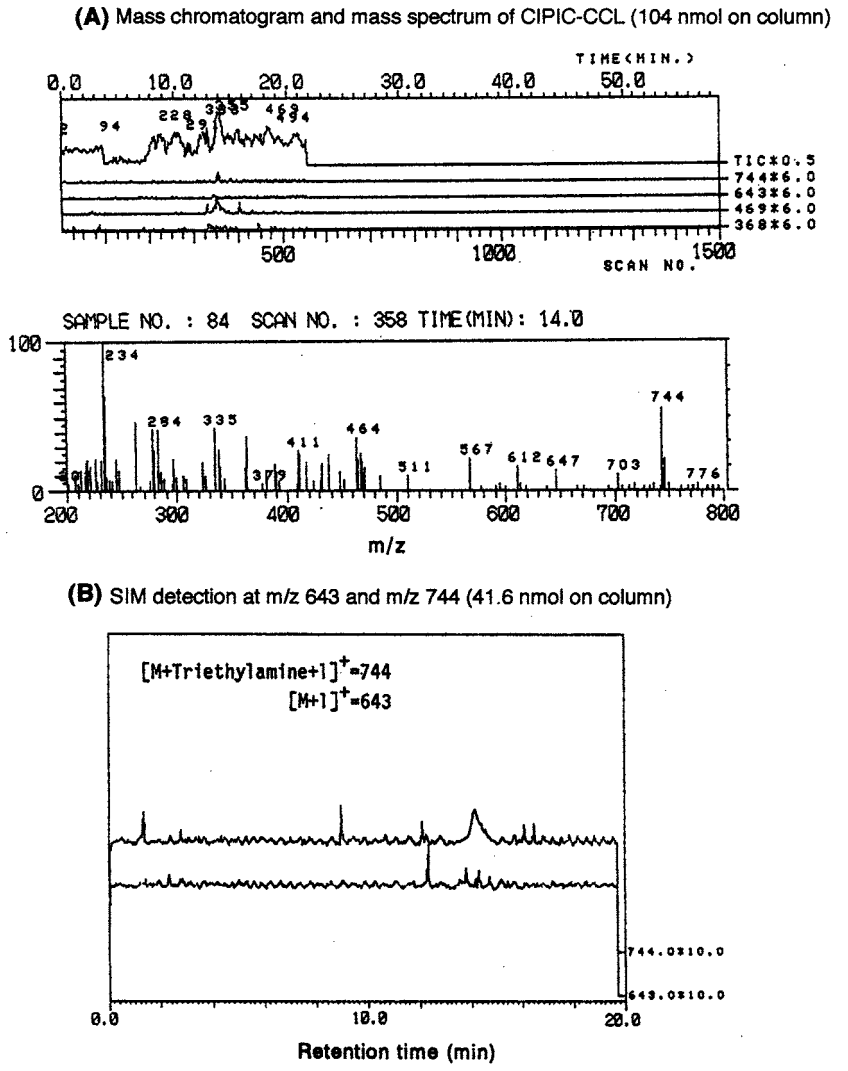

Fig. 9. LC/MS of a reaction mixture of CCL with CIPIC.

emission and excitation wavelengths of 410 and 310 nm, respectively. The FL peak of CIPIC-CCL in the chromatogram corresponded to its CL peak (Fig. 4). The sensitivity of the FL detection was ca. 10 times lower than that of the CL detection. The lower detection limit of the CCL–CIPIC compound was 1.0 pmol by the 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. A maximum intensity at pH 9.6 was obtained at ca. 90 s after initiating the reaction (Fig. 6). In this HPLC detection system, thus the column eluate was passed through a stainless coil

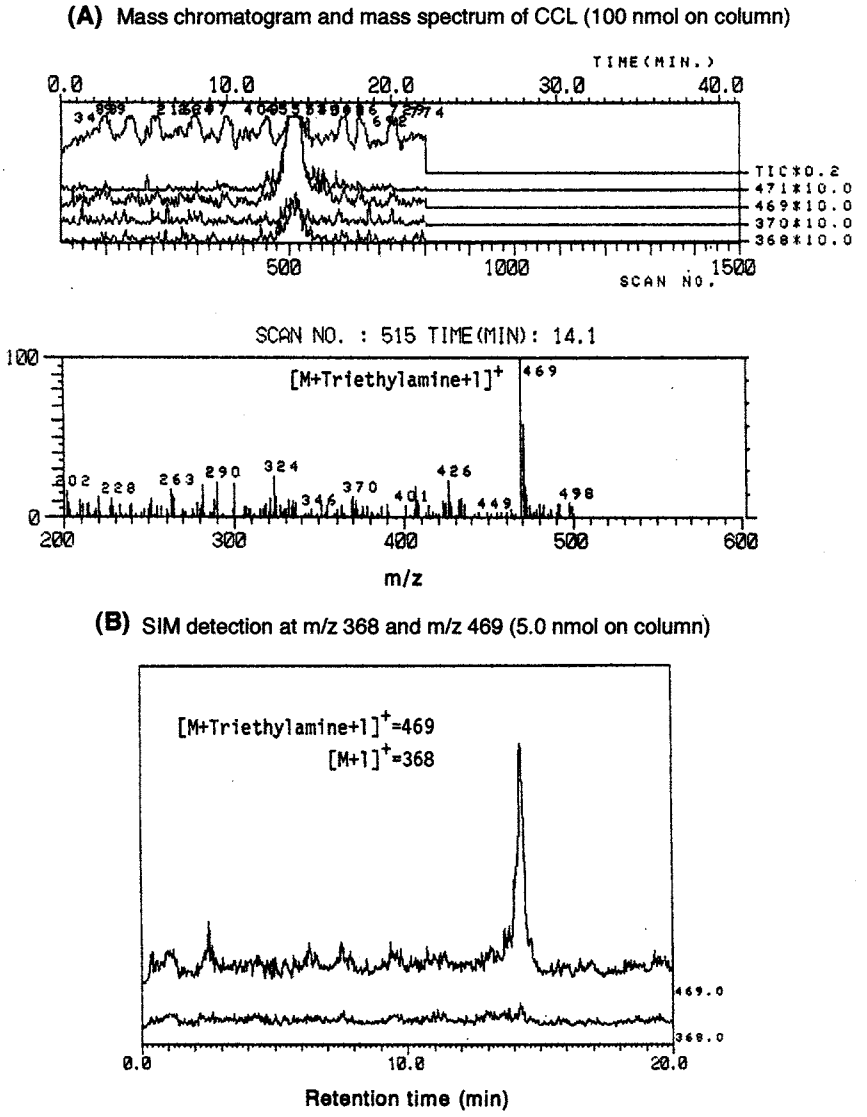

Fig. 10. LC/MS of authetic CCL.

(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 ca. 50% (v/v) acetonitrile. The CL intensity from CIPIC-CCL resulted in ca. sixfold increase as compared with that obtained without acetonitrile in the post-column reagent solutions of borate buffer and H_2O_2.

3.3. Determination of serum CCL

Human sera were only deproteinized by denaturation. This denaturation was conducted by heating the samples during the derivatization reaction. Thus, this pretreatment for the biological samples was very simple. There was no significant loss of CCL in serum by the reaction, since the CCL recovery was $100 \pm 6\%$ ($n = 8$), as compared without serum. 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 was very similar to that of CCL (Fig. 1).

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) was 6.9–7.0% for the ratio and 19.5–13.1% for the peak height. These 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-conjugated CCL and/or by the error of its injected volume into the chromatogram. Therefore, the calibration graphs for the FL and CL determinations were made, respectively by plotting the peak height ratio against the concentration of CCL in serum (0, 2.5 5, 10, 15, 20, 25 μM, $n = 2$ for each). The regression equation for the graph was $Y = 0.282X - 0.162$ by the FL detection, and $Y = 0.261X + 0.229$ 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 the oral administration of CCL to a subject at 30 min after breakfast is shown in Fig. 8. Its half-life in serum after the administration was 1.04 h. In other report [3], a maximum concentration of CCL in serum after oral administration (500 mg) to fasting subjects was ca. 34 μM, and its half-life time was ca. 0.7 h. In the comparison, the maximum concentration in Fig. 8 was about half of that found in the fasting subjects, since the maximum concentration in the case with food was decreased to 17–35 μM, and the peak concentration is generally achieved about 1 h later [3].

3.4. MS detection

The quasi-molecular ions $[M + \text{triethylamine} + H]^+$ at $m/z = 744$ and 469 of the CIPIC-conjugated CCL and non-modified CCL were detected, respectively by LC/MS analysis, as shown in Fig. 9A and Fig. 10A. It suggests that the quasi-molecular ion attached with triethylamine was yielded because of the presence of triethylamine of the mobile phase in the APCI interface of the MS instrument. Thus we tried to quantitatively determine the compounds by the LC/MS using selected-ion monitoring (SIM) mode (Fig. 9B and Fig. 10B). The SIM detection of LC/MS could make possible to determine both compounds, though the detection limits (S/N = 3) were ca. 10 nmol for CIPIC-CCL and 0.5 nmol for CCL.

This study demonstrated MS, FL and CL methods utilizing the derivatization technique with CIPIC. The use of CIPIC was the first time for the derivatization of CCL. The proposed HPLC method coupled with FL and CL detections could be applied to the quantitative determination of CCL in human serum. This HPLC protocol permits the assay of the CCL serum concentrations higher than 1 μM by the FL detection, and 0.1 μM by the CL detection. The results also provide 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. The LC/MS analysis was useful for the confirmation of the chemical structure of the CIPIC-conjugated CCL. However, the MS instrument unexpectedly showed lower sensitivity than the FL and CL detections.

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