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Short Communication

Determination of aspoxicillin in broncho-alveolar lavage fluid by high-performance liquid chromatography with photolysis and electrochemical detection

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ABSTRACT

A determination method for the penicillin antibiotic aspoxicillin in broncho-alveolar lavage fluid has been developed, involving high-performance liquid chromatography and post-column photolysis. The method enabled the determination of aspoxicillin at concentrations below 100 ng/ml, with a linear calibration curve at concentrations of 1–1000 ng/ml for 100 μl of lavage fluid. The detection limit was 1 ng/ml at a signal-to-noise ratio of 3:1.

INTRODUCTION

Distribution profiles of antibiotics in infected tissues have great importance in selecting a drug and its dosage regimen. Broncho-alveolar lavage fluid (BALF) gives a valuable clue to the treatment of respiratory infections. To determine levels of aspoxicillin (ASPC) in BALF, established high-performance liquid chromatographic (HPLC) [1] or bioassay [2] methods are often impractical when low concentrations or concomitant use of other antibiotics were encountered.

Various highly sensitive pre- or post-column HPLC methods have been reported for the separation and determination of penicillin antibiotics in body fluids. Among the pre-column methods are the formation of fluorescent degradation products in the presence of mercury(II) chloride after alkaline degradation and acid hydrolysis [3], and a derivatization technique using 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole [4]. The post-column methods include those using reagents such as fluorescamine [5], imidazole mercuric chloride [6] or alkaline mercuric chloride [7]. A pre-column HPLC method consisting of acid or alkaline degradation, tautomerization with mercuric chloride and fluorometric detection has been re-

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ported for ampicillin in BALF [8]. However, the methods mentioned above required toxic mercuric chloride as a catalyst, therefore, the development of a safer method with a wide use has been sought.

Selavka *et al.* [9] have recently demonstrated a highly sensitive post-column HPLC method for β-lactams using a photochemical reaction system. This system involved photochemical degradation of the β-lactam and determination of its degradation product by electrochemical detection (ED). Krull *et al.* [10] have discussed many aspects of such photoderivatization methods in HPLC.

However, application of these photochemical ED methods to a variety of body fluids has not been fully investigated. We applied this principle to the assay of ASPC in BALF and established an analytical method enabling the determination of low concentrations of ASPC. Our method consists of the following procedures: separation of ASPC by HPLC, photolysis of ASPC and the measurement of the oxidation current by ED.

EXPERIMENTAL

Broncho-alveolar lavage fluid

The bronchi and alveoli were perfused twice 60 min after intravenous administration of 1 g of ASPC to a patient, each with 50 ml of sterilized physiological saline solution and aspirated. The fluids were pooled and stored at −20°C until assay.

Chemicals

ASPC (Fig. 1) was supplied by Tanabe Seiyaku (Osaka, Japan). Amoxicillin (AMPC), used as an internal standard, was from Sigma (St. Louis, MO, USA). Sodium 1-heptanesulphonate, for use in paired-ion HPLC, was from Nacalai Tesque (Kyoto, Japan). Potassium dihydrogenphosphate and phosphoric acid were from Wako (Osaka, Japan). Disodium ethylenediaminetetraacetic acid (EDTA) was from Dojindo Labs. (Kumamoto, Japan). These were of special analytical grade. Acetonitrile, for HPLC use, was from Katayama Kagaku (Osaka, Japan). Distilled water for HPLC was from Wako.

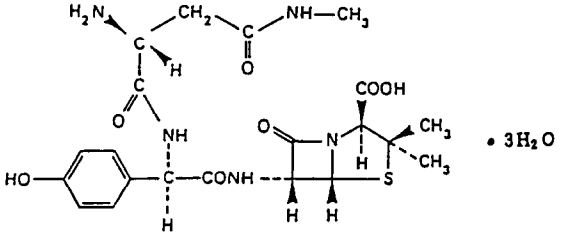

Fig. 1. Structure of ASPC.

Apparatus

A Kontron HPLC system (Kontron, Milan, Italy), consisting of a Model 420 pump, a Model 460 automatic sample injector, a Model 480 column oven, a Model 430 UV detector and a Model 450 system controller and data-processing unit, was equipped with a Σ08 photo deriver or photochemical reactor (Irica, Kyoto, Japan) and a Σ875 amperometry electrochemical detector (Irica). The automatic sample injector was cooled with an RTE 110 cooling unit (Neslab, Newington, NH, USA) at 5°C. Reversed-phase HPLC separations were performed using a Shodex C_{18} 5A column (150 mm × 4.6 mm I.D., particle size 5 μm; Showa Denko, Tokyo, Japan).

The photochemical reactor was supplied with a GL-10 tubular 10-W mercury lamp (Toshiba, Tokyo, Japan). This light source emits an intense band at 254 nm. PTFE tubing (10 m × 0.3 mm I.D.) was wound around the mercury lamp. Irradiation of the HPLC eluent took place within the PTFE tubing. The UV detector was coupled to the outlet of the analytical column, followed by the photoreactor and the electrochemical detector. Ultracent-30, an ultrafiltration membrane filter (Tosoh, Tokyo, Japan), had a molecular mass cut-off at 30 000.

Analytical procedure

To 1 ml of BALF samples, 100 μl of 5 μg/ml AMPC (the internal standard) solution were added in a 10-ml glass tube. The mixture was vortex-mixed for 10 s and then ultrafiltered, using Ultracent-30, at 1500 *g* for 30 min at 5°C. A 100-μl portion of the ultrafiltrate was applied to the

HPLC system. Other conditions for analysis are shown in Table I.

Calibration curve

A calibration curve was constructed by plotting the peak-height ratio of ASPC to the internal standard *versus* the concentration.

Precision and accuracy

The precision of the method was calculated as the coefficient of variation (C.V.) of replicate assays of standards containing ASPC at concentrations from 1 to 100 ng/ml BALF.

RESULTS AND DISCUSSION

Photochemical detection of ASPC

The HPLC chromatograms obtained from a concentration of 250 ng each of ASPC and AMPC standard are shown in Fig. 2. The electrochemical detector had a single working electrode. To confirm the analyte peak, the UV detector was connected to the outlet of the analytical column, followed in series by the photoreactor and the electrochemical detector.

When the lamp was off, only the UV detector responded to the analytes and no response was recorded on the electrochemical detector. This demonstrated that both antibiotics lack detectable oxidative properties, as reported for other β-lactam antibiotics [9,10]. When the lamp was on, the electrochemical detector showed two large peaks, which corresponded to those peaks on the UV detector with a lag time of 1.5 min, thus supporting specificity of this detection method.

Selavka *et al.* [9] and Krull *et al.* [10] have monitored the current response ratio by using dual working electrodes to improve the selectivity of their photochemical derivatization method. Although our system was equipped with a less specific single-electrode detector, the lamp on/off study as well as the serial detection support the selectivity of the analysis.

Chromatographic separation conditions

A mobile phase of a 0.05 *M* potassium phosphate buffer containing 0.02 *M* sodium 1-heptanesulphonate and 5 mg/l EDTA (final pH 3.0)–acetonitrile (100:10) was found to be effective in separating ASPC, AMPC and the impurities in BALF. Fig. 3 shows typical chromatograms obtained with ED. The use of acetonitrile as a mobile phase constituent had previously led to problems, namely the rapid destruction of the helical PTFE reactor [11] and fouling of the electrode by polymerization of acetonitrile [10]. Under our experimental conditions, however, no such inconvenience was observed in continual experiments for at least two weeks.

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR MEASUREMENT OF ASPC

Analytical column	Shodex C_{18} 5A 5 μm, 150 mm × 4.8 mm I.D.
Mobile phase	20 m*M* Sodium 1-heptanesulphonate, 5 mg/l EDTA in 0.05 *M* potassium hydrogenphosphate (pH 3.0)–acetonitrile (100:10, v/v)
Flow-rate	1.2 ml/min
Column temperature	40°C
UV detection wavelength	230 nm
Electrochemical detection	
Working electrode	Glassy carbon
Reference electrode	Ag/AgCl
Time constant	Standard
Applied voltage	800 mV

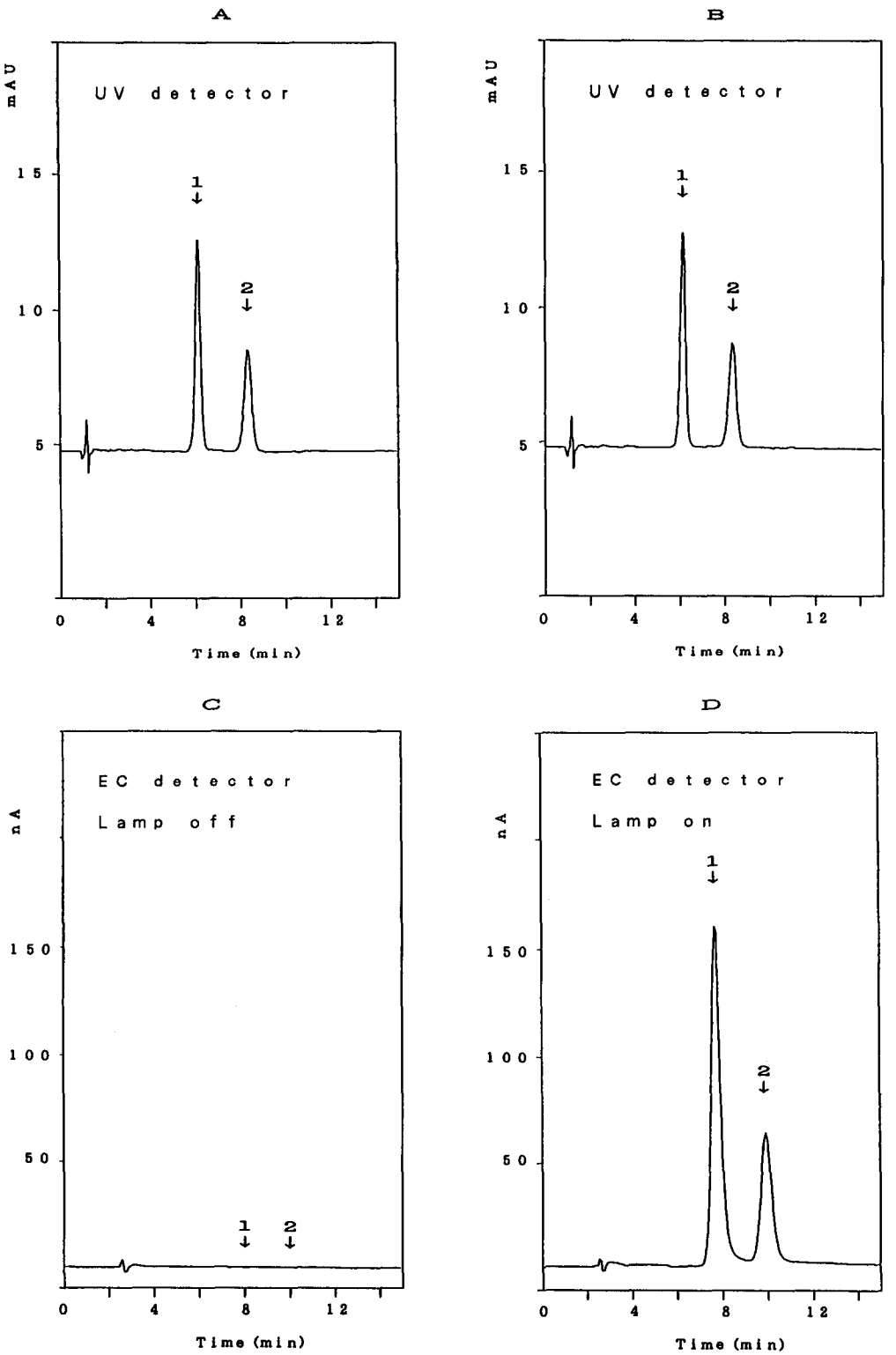

Fig. 2. Chromatograms obtained from 250 ng each of ASPC and AMPC. The UV detector was connected to the column outlet followed by the photoreactor and the electrochemical detector. After ASPC and AMPC were detected by the UV detector with the lamp off (A) or on (B), each eluent was monitored by ED successively. There was no peak when the lamp was off (C), whereas large peaks appeared when the lamp was on (D). Peaks: 1 = AMPC; 2 = ASPC.

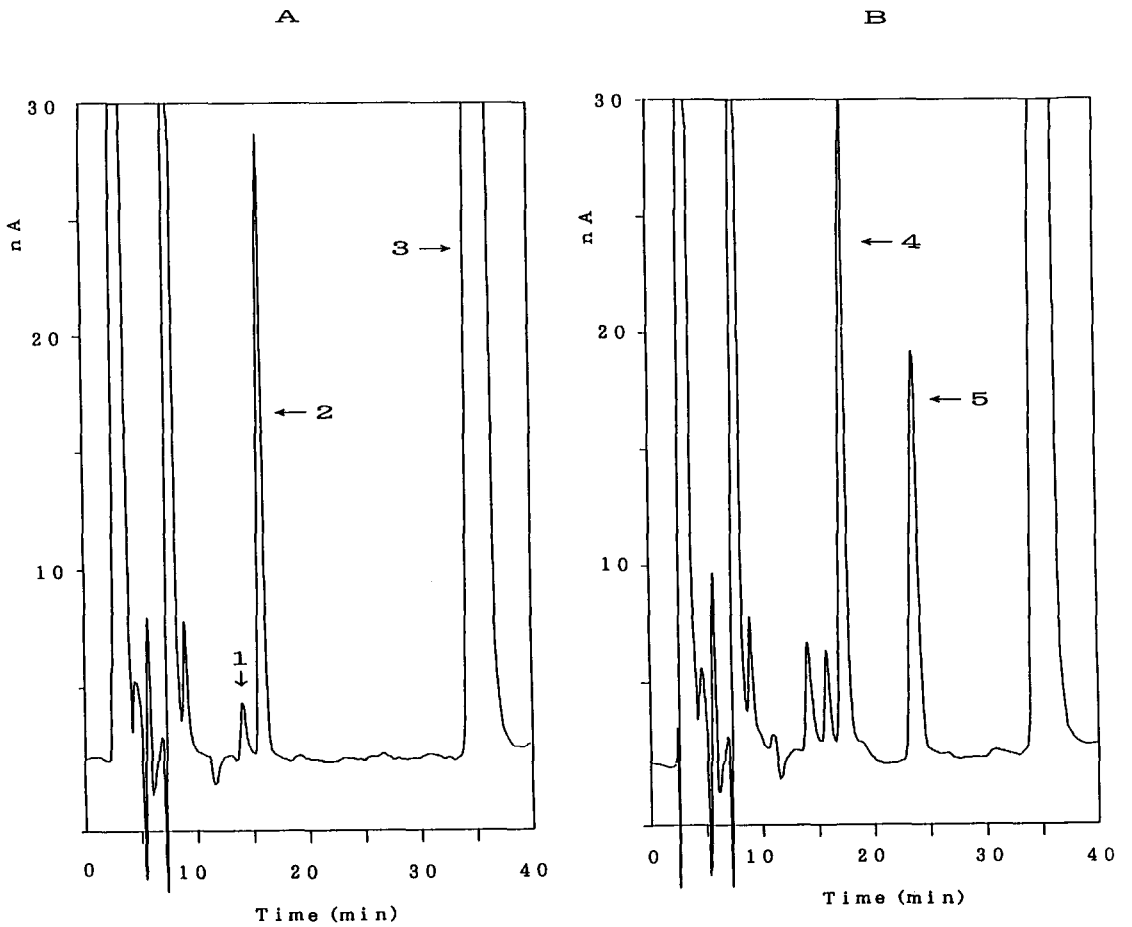

Fig. 3. Chromatographic separation of ASPC and AMPC from impurities in BALF, with photoreaction and electrochemical detection. A mobile phase of a 0.05 *M* potassium phosphate buffer containing 0.02 *M* sodium 1-heptanesulphonate and 5 mg/l EDTA (final pH 3.0)–acetonitrile (100:10) was used. (A) Blank BALF; (B) BALF spiked with ASPC and AMPC (500 ng/ml each). Peaks: 1, 2 and 3 = impurities from BALF; 4 = AMPC; 5 = ASPC,

Post-column reactor and detector conditions

Optimization of PTFE tubing length. The ED response reached a plateau at a PTFE tubing length of 10 m. Slight degradation of ASPC was observed at lengths of 15 m or longer. Little change was observed in the peak width at half height with lengths between 2 and 20 m. Thus, 10 m of PTFE tubing was used for the final reactor.

Hydrodynamic voltametry. Hydrodynamic voltametry was performed under the conditions above described to select an appropriate applied voltage for ED. The ED response to both ASPC and AMPC rapidly increased at 700 mV or more. Thus, 800 mV was used as the applied voltage.

Calibration curve

Under the measurement conditions selected, a calibration curve for ASPC was obtained. It was linear over a range of 1–1000 ng/ml with a regression of $y = 0.068x - 0.0468$, and a correlation coefficient of 0.996, where y represents the peak-height ratio of ASPC to the internal standard and x is the concentration of ASPC in ng/ml. The detection limit was 1 ng/ml at a signal-to-noise ratio of 3:1.

Precision and accuracy

Table II shows the precision of the assay. The C.V. of within-day and day-to-day assays were

TABLE II

PRECISION AND ACCURACY OF THE ASSAY IN SPIKED BRONCHO-ALVEOLAR LAVAGE FLUID SAMPLES

Quantity added to 1 ml BALF (ng)	Within-day (n = 5)			Day-to-day (n = 4)		
	Mean estimated (ng/ml)	Accuracy (%)	C.V. (%)	Mean estimated (ng/ml)	Accuracy (%)	C.V. (%)
1	1.14	114	22.7	1.03	103.3	11.4
10	10.2	101.6	20.8	9.24	92.4	17.7
100	94.9	94.9	7.5	84.2	84.2	9.8
1000	947	94.7	3.2	932	93.2	3.1

3.2–22.7% (n = 5) and 3.1–17.7% (n = 4), respectively. The recovery was between 84.2 and 114%.

Analysis of clinical BALF samples

ASPC concentrations were determined in BALF from patients who had received 1 g of ASPC. Fifteen BALF samples were collected 60 min after the injection. The concentrations ranged from 59 to 284 ng/ml, with a mean value of 146 ng/ml; these are within the practical range of determination.

The sequential determination by UV detection and ED revealed that the values determined by photoreaction and ED correspond well with those determined by UV detection for six samples in which the concentration exceeded 160 ng/ml. This suggested that the electrochemical detector did detect an analytical signal derived from intact ASPC rather than any potential metabolite in the BALF.

CONCLUSION

In our investigation, the effect exerted by a photoreactor was first confirmed by using a UV detector–photoreactor–electrochemical detector; the mobile phase of 0.05 M phosphate buffer (pH 3.0)–acetonitrile (100:10) containing 0.02 M sodium 1-heptanesulphonate and 5 mg/l EDTA was selected to separate ASPC from the other substances, including impurities in BALF and AMPC. For the post-column conditions, 800 mV of ED applied voltage was selected as the applied voltage based on the responses. These conditions (summarized in Table I) enabled the determination of ASPC at levels as low as 1 ng/ml. This method thus proved to be more sensitive than any previous method for low concentrations of ASPC. Moreover, it requires no more than a pretreatment by ultrafiltration to analyse clinical samples. This method is, therefore, considered useful for examining the distribution profile of ASPC in BALF.

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