Journal of Chromatography, 532 (1990) 87–94
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam

CHROMBIO. 5428

Simultaneous determination of ampicillin and sulbactam by liquid chromatography: post-column reaction with sodium hydroxide and sodium hypochlorite using an active hollow-fibre membrane reactor

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(First received April 3rd, 1990, revised manuscript received May 22nd, 1990)

ABSTRACT

A high-performance liquid chromatographic method has been developed for the simultaneous determination of ampicillin (ABPC) and sulbactam (SBT) in serum and urine The method involves separation of ABPC and SBT from the background components of serum and urine on a C_{18} column, post-column reaction with sodium hydroxide and sodium hypochlorite using an active hollow-fibre membrane reactor, and detection at 270 nm At ABPC and SBT concentrations of 10 and 5 μg/ml in urine and serum samples, the precisions (relative standard deviations) were 0 9–2 5% (n = 8) The detection limits were 20 and 5 ng for ABPC and SBT, respectively, at a signal-to-noise ratio of 3

INTRODUCTION

The identification of β-lactamase-producing bacteria has led to the development of new drug derivatives that are resistant to β-lactamases. Also, β-lactamase inhibitors are used to potentiate the activity of β-lactamase-susceptible penicillins [1]. Potassium clavulanate formulated with amoxicillin or ticarcillin and sodium sulbactam (SBT) formulated with ampicillin (ABPC) have been recently made available in Japan for clinical use.

These penicillins and β-lactamase inhibitors have no accessible UV absorption maxima for detection following high-performance liquid chromatography (HPLC). Previously, we reported HPLC methods for the determination of these substances in serum and urine, which employed post-column reaction with sodium hypochlorite and sodium hydroxide for penicillins [2–4] and sodium hydroxide for β-lactamase inhibitors [5]. In these methods the reagents were delivered by a pump and mixed with the eluent through tees. We subsequently found considerable improvements in all aspects of system operation and limits of detection when the same reagents were introduced through cation- and/or anion-exchange membrane reactors immersed in the reagent solutions [6–8]. The membrane in-

0378-4347/90/$03.50

troduction technique not only dispenses with a reagent-delivery pump, but also eliminates sample dilution, minimizes chromatographic dispersion, produces baseline noise levels far better than those obtained with the tee introduction and provides even better reproducibility [9,10]

Post-column reaction systems based on the membrane introduction technique can be divided into two broad types, passive and active [11]. In a passive membrane system, a permeable membrane such as an ion-exchange membrane immersed in the concentrated reagent solution is used, as described above. The concentration gradient of the reagent between the two sides of the membrane allows the reagent to permeate into the inner flow stream. In an active membrane system, a porous membrane is used, and the reagent is introduced into the inner flow stream through the membrane walls by pressure.

We have not previously investigated the simultaneous determination of penicillins and β-lactamase inhibitors. This paper describes an HPLC method for the simultaneous determination of ABPC and SBT in serum and urine using an active hollow-fibre membrane (HFM) reactor.

EXPERIMENTAL

Reagents and materials

Sodium SBT was kindly donated by Kanebo (Osaka, Japan). Sodium ABPC and tetrabutylammonium bromide (TBAB) were purchased from Nacalai Tesque (Kyoto, Japan). Control serum (Control Serum I Wako) and other reagents of analytical grade were obtained from Wako (Osaka, Japan). An active HFM reactor (Membrane Reactor 1) was obtained from Dionex (Sunnyvale, CA, U.S.A.).

Water purified by a Nanopure II unit (Barnstead, Boston, MA, U.S.A.) was used for the preparation of eluent and sample solutions.

Chromatography

The experimental set-up is illustrated in Fig. 1. The instrumentation was as follows: an 880-PU pump; a VL-614 loop injector equipped with a 100-μl loop (both from Japan Spectroscopic, Tokyo, Japan); a main column (250 mm × 4 mm I.D.) and a guard column (4 mm × 4 mm I.D.) packed with LiChrospher RP-18(e) (particle size 5 μm; E. Merck, Darmstadt, F.R.G.); an active HFM reactor (400 mm × 0.5 mm I.D) for introducing a reagent solution into the inner flow stream; a knitted open tubular (KOT) reactor (140 cm × 0.3 mm I.D.) constructed from PTFE tubing for complete mixing of the eluent and reagent and increasing the reaction time; a thermostatted water-bath (Lauda RMS 6, Hansen, Kobe, Japan) set at 50°C; a UVIDEC 100-V detector (Japan Spectroscopic); and a Chromatopak C-R6A (Shimadzu, Kyoto, Japan) for recording and integrating chromatographic peaks. The active HFM reactor was used with a coil geometry, and the KOT reactor used was knitted according the procedures reported by

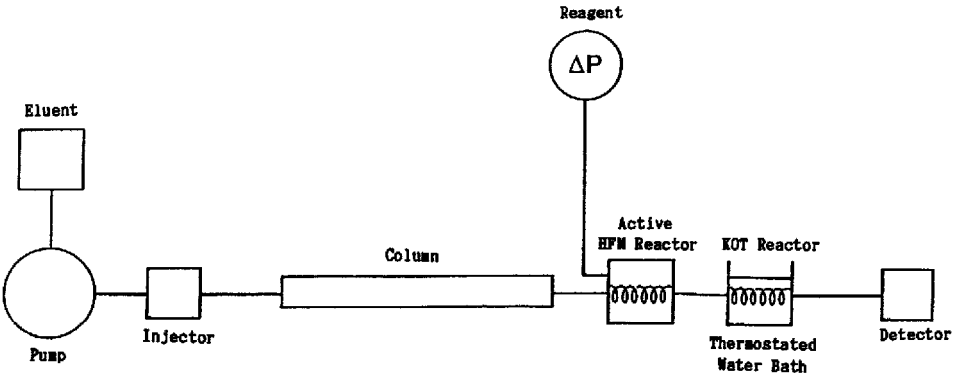

Fig. 1 Experimental set-up for the simultaneous determination of ABPC and SBT The reagent was introduced through an active HFM reactor (400 mm × 0 5 mm I D.) into the main flow stream by nitrogen pressure, and a KOT reactor (140 cm × 0.3 mm I.D.) immersed in a thermostatted water-bath was used as a delay coil.

Selavka *et al.* [12] (KOT2 reactor). When the internal nitrogen pressure was set to 2.7 bar at a reaction temperature of 40°C, the volume of reagent solution introduced into the main flow stream was equivalent to 0.1 ml/min. The eluent used was 20 m*M* TBAB, 5m*M* sodium dihydrogenphosphate plus 5 m*M* disodium hydrogenphosphate–methanol (1.75:1, v/v) for the separation of ABPC and SBT in urine and serum samples. The flow-rate was maintained at 0.8 ml/min. Detection was performed at 230 and 270 nm, respectively, without and with post-column reaction.

Measurement of void volume and band broadening

A 20-μl portion of $8 \cdot 10^{-4}$ *M* potassium iodate was injected on a flow line delivering water at a flow-rate of 0.8 ml/min, and an elution peak was monitored at 254 nm. The dead volume (V_0) and band broadening (σ_v) of the active HFM and KOT reactors were calculated with and without each reactor.

Sample preparations

For serum samples, ABPC and SBT standards were dissolved in human control serum. The serum samples were ultrafiltered by using Molcut II (Nihon Millipore, Tokyo, Japan). A 50-μl portion of the ultrafiltrate was loaded onto a column. For urine samples, ABPC and SBT standards were dissolved in human control urine. The urine samples were diluted ten-fold with water and filtered with a 0.45-μm acrylate copolymer membrane (Gelman Science Japan, Tokyo, Japan). A 20-μl portion of the filtrate was loaded onto the column.

RESULTS AND DISCUSSION

Post-column reaction conditions

Previously, we reported that the degradation of SBT by sodium hydroxide in the presence of methanol yielded methyl 5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoate, which has a UV absorption maximum at *ca.* 280 nm [13]. ABPC was converted into methyl ampicilloate in the presence of methanol (which does not have a UV absorption maximum at *ca.* 260 nm) and further degraded to a product(s) in the presence of sodium hypochlorite, which has a UV absorption maximum at *ca.* 260 nm [2]. Thus, an HPLC method based on post-column degradation reaction with sodium hydroxide and sodium hypochlorite with an active HFM reactor was investigated for the simultaneous determination of ABPC and SBT.

First, an active HFM reactor only was used. The baseline noise due to the incomplete mixing of eluent and reagent prevented sensitive detection of ABPC and SBT. Thus, the KOT reactor was connected in series with the active HFM reactor. A 50-μl portion of ABPC and SBT standard solutions (each 10 μg/ml) was loaded onto the column, and the peak height was measured. The length of the active HFM reactor used was 400 mm, and the volume of reagent solution introduced into the main flow stream was kept constant at 0.1 ml/min by varying the internal nitrogen pressure.

The reaction conditions were examined with respect to concentrations of sodium hydroxide and sodium hypochlorite, the length of the KOT reactor and the reaction temperature. Figs. 2 and 3 show the effect of different concentrations of sodium hydroxide and sodium hypochlorite on the UV absorbance of the degradation products of ABPC and SBT. The response of SBT decreased with an increase of the concentration of sodium hypochlorite and/or a decrease of the concentration of sodium hydroxide. The reverse tendencies were observed with the response of ABPC. Thus, the best concentrations of sodium hydroxide and sodium hypochlorite were determined to be 2 *M* and 0.05%, respectively. Fig. 4A and B show the effects of reaction temperature and length of the KOT reactor on the peak heights of SBT and ABPC, respectively. The response of SBT was maximal at a reaction temperature of 30°C and a KOT reactor length of 140 cm and at a reaction temperature of 50°C and a KOT reactor length of 60 cm; ABPC gave a maximal response at a reaction temperature of 50°C and a KOT reactor length of 140 cm. These results suggest that the degradation of SBT proceeds faster than that of ABPC. Thus, the reaction temperature and KOT reactor length were set at 50°C and 140 cm for the simultaneous determination of ABPC and SBT.

Band broadening due to the post-column reactor

Table I lists the void volumes (V_0) of the active HFM and KOT reactors and the band broadening (σ_v) in each reactor. Despite the larger void volume of the

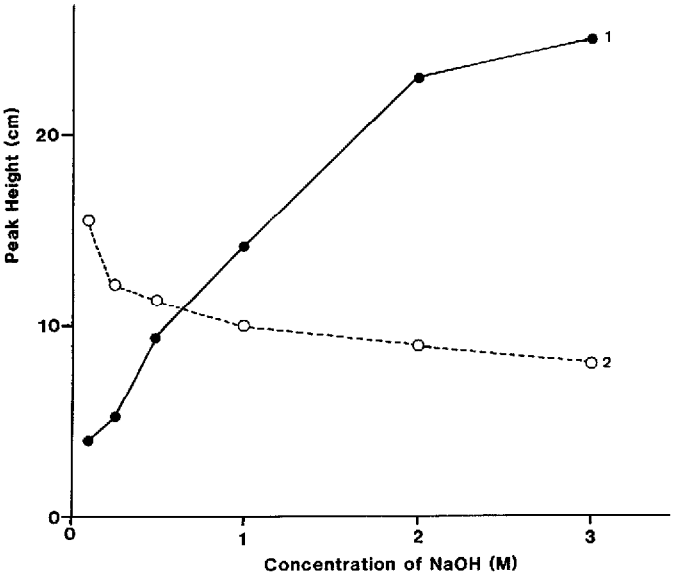

Fig 2 Effect of the concentration of sodium hydroxide on the UV response of the degradation product(s) of SBT (1) and ABPC (2). Other parameters were kept constant. concentration of sodium hypochlorite, 0 05%, reaction temperature, 50°C, length of KOT reactor, 140 cm.

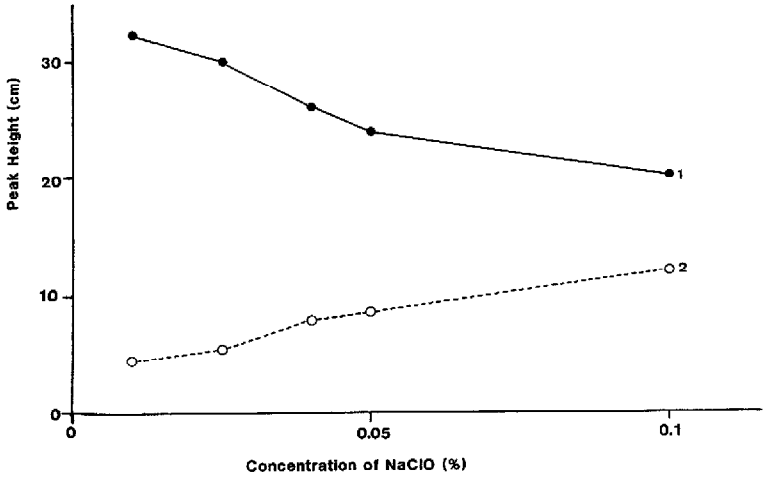

Fig. 3 Effect of the concentration of sodium hypochlorite on the UV response of the degradation product(s) of SBT (1) and ABPC (2). Other parameters were kept constant· concentration of sodium hydroxide, 2 *M*; reaction temperature, 50°C, length of KOT reactor, 140 cm.

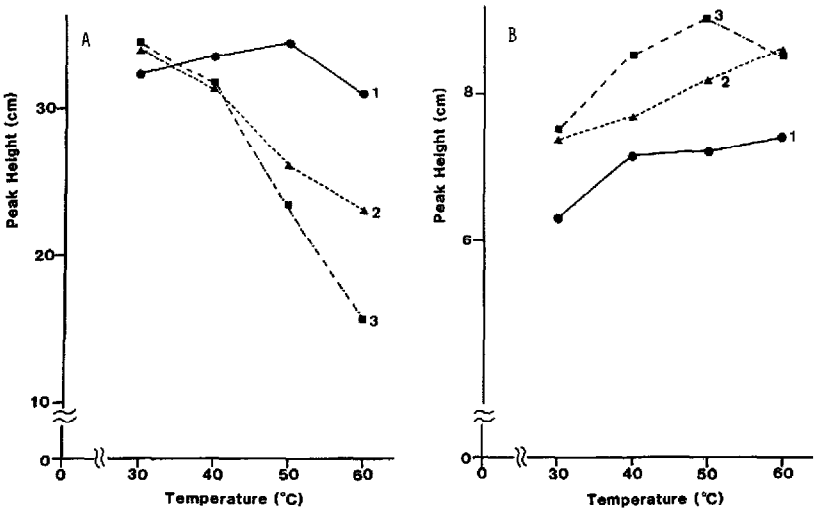

Fig. 4. Effect of reaction temperature and length of KOT reactor on the UV response of the degradation product(s) of (A) SBT and (B) ABPC. Length of KOT reactor: 1, 60 cm; 2, 100 cm, 3, 140 cm. Other parameters were kept constant: concentrations of sodium hydroxide and sodium hypochlorite, 2 *M* and 0.05%, respectively

KOT reactor, its band broadening was smaller than that of the active HFM reactor. This is due to the differences in the reactor geometries and inside diameters [12].

Application to simultaneous determination of ABPC and SBT in serum and urine

On the basis of these results, we applied the method to the simultaneous determination of ABPC and SBT in serum and urine. Figs. 5 and 6 show chromatograms of ABPC and SBT in serum ultrafiltrate and ten-fold diluted urine, respectively. The two substances were well separated from the background components of serum and urine samples, with TBAB used as an ion-pairing agent. ABPC and SBT were detected at 270 nm following post-column reaction 1.2 and

TABLE I

DEAD VOLUME (V_0) OF ACTIVE HFM AND KOT REACTORS AND BAND BROADENING (σ_v) IN THE POST-COLUMN REACTOR

Reactor	V_0 (μl)	σ_v (μl)
HFM	86	30
KOT	136	21
Total	222	36

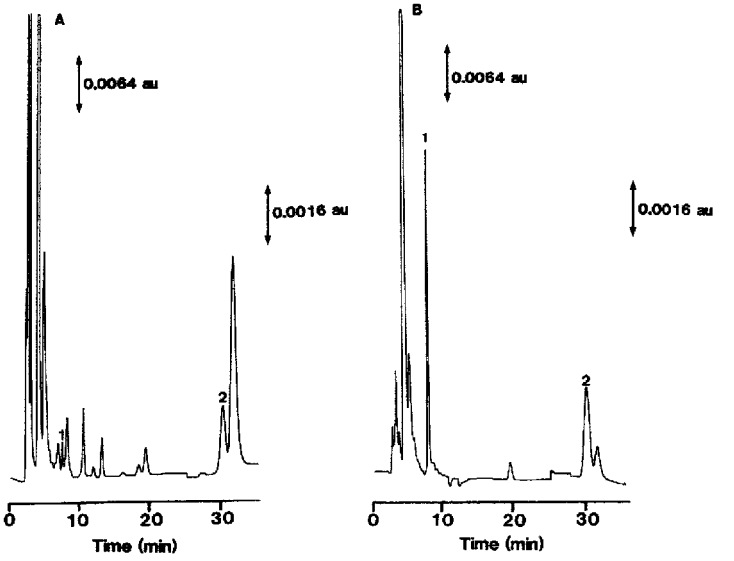

Fig 5. Separation of SBT and ABPC from the background components of serum ultrafiltrate A 50-μl portion of the ultrafiltrate of serum samples was loaded onto the column. (A) Detection at 230 nm without post-column reaction, (B) detection at 270 nm with post-column reaction. Concentrations SBT, 5 μg/ml, ABPC, 10 μg/ml Other conditions are given in the text. Peaks 1 = SBT, 2 ABPC.

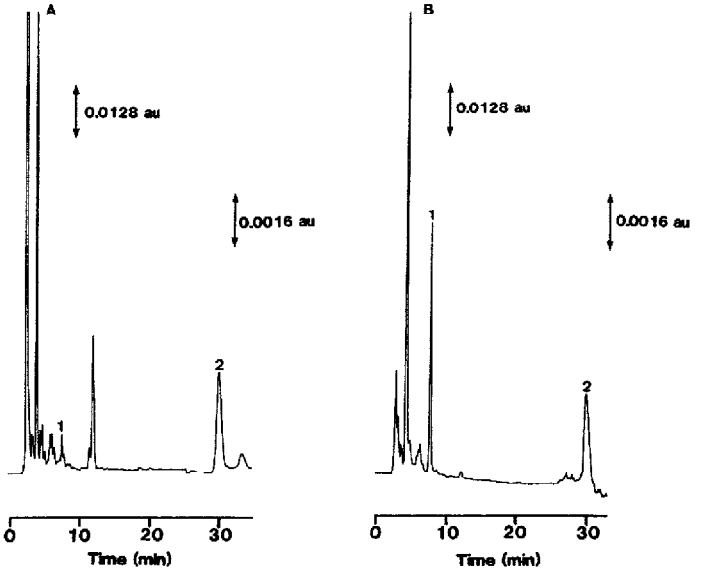

Fig. 6. Separation of SBT and ABPC from the background components of ten-fold diluted urine. A 20-μl portion of the filtrate of ten-fold diluted urine samples was loaded onto the column. (A) Detection at 230 nm without post-column reaction; (B) detection at 270 nm with post-column reaction Concentrations 20 μg/ml each after ten-fold dilution Other conditions are given in the text. Peaks 1 = SBT; 2 = ABPC

10 times more sensitively compared with detection at 230 nm. Further, detection at 270 nm was free of interferences from background components, and repeated injection was possible.

Reproducibility, linearity and detection limit

At ABPC and SBT concentrations of 10 and 5 μg/ml in serum and urine samples, the precisions (relative standard deviations) were 0.9–2.5% ($n=8$). The detection limits for ABPC and SBT were 20 and 5 ng, respectively, at a signal-to-noise ratio of 3. The calibration graphs of peak height *versus* concentration for ABPC and SBT in serum ultrafiltrate and ten-fold diluted urine were linear in the concentration range 1–40 μg/ml, with a correlation coefficient of 0.999 or above, and passed through the origin.

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