[Chem. Pharm. Bull. 32(7)2752—2758(1984)]

Sulbactam: Alkaline Degradation and Determination by High-Performance Liquid Chromatography

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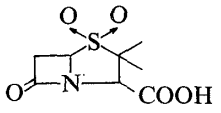
(Received October 22, 1983)

Although sulbactam has no ultraviolet (UV) absorption maximum above 200 nm, it was found to be easily degraded in alkaline methanol and in alkaline aqueous solution to yield products having UV absorption maxima at 267 nm and 278 nm, respectively. An ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method utilizing this alkaline degradation as a postcolumn reaction has been developed for the determination of sulbactam in plasma and urine. A high and reproducible detector response due to the degradation product(s) was obtained when methanol was used as an organic modifier of the eluent. The HPLC and postcolumn reaction conditions were as follows: column, Develosil ODS-10 (25 cm × 4.6 mm i.d.); eluent, 5 mM tetra-*n*-butylammonium bromide + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/methanol = 3/1 (v/v) for urine samples and 2/1 (v/v) for plasma samples; flow rate, 1.2 ml/min; postcolumn reagent, 0.5 N aqueous NaOH solution at a flow rate of 0.6 ml/min; reaction coil, 2 m × 0.25 mm i.d.; detection, 276 nm. The procedure was quantitative over a wide range of sulbactam concentrations in plasma and urine down to 0.2 μg/ml and 1.0 μg/ml, respectively. The intra- and inter-assay precisions for plasma at a sulbactam level of 0.53 μg/ml were of the order of 2.0% and 3.5%, respectively.

Keywords——sulbactam; sulbactam HPLC; sulbactam alkaline degradation; postcolumn derivatization; beta-lactamase inhibitor; penicillanic acid sulfone; CP-45899

One way to restore the susceptibility of resistant strains of β-lactamase-producing bacteria to β-lactam antibiotics is the combined use of the antibiotics with β-lactamase inhibitor. Many β-lactamase inhibitors have been isolated or synthesized. Sulbactam (penicillanic acid sulfone, CP-45899), (2*S*, 5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (I), is a potent semi-synthetic β-lactamase inhibitor.[1)]



I

Sulbactam is somewhat less potent in inhibitory activity, but is much more stable, than clavulanic acid isolated from *Streptomyces clavuligerus* ATCC 27064.[1-3)] Various *in vitro* and *in vivo* experiments revealed that the combined use of sulbactam with a certain class of penicillins or cephalosporins is effective against various β-lactamase-producing bacteria.[1-8)]

Sulbactam has been determined mainly by using a microbiological method[9)] which has been routinely used for the determination of β-lactam antibiotics. Baltzer *et al.*[10)] reported a gas chromatographic method for the determination of sulbactam in urine and plasma. However, the method required tedious extraction and derivatization procedures. Rogers *et*

al.[11] reported a high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection at 225 nm for assays of sulbactam in plasma, urine and saliva. However, an extraction procedure was necessary, and the extraction efficiency was as low as 55%. A simple and rapid assay method is required to assess the purity of sulbactam in pharmaceutical preparations and to investigate the pharmacokinetic behavior of the inhibitor.

In the previous paper,[12] we reported that an HPLC method with postcolumn alkaline degradation was useful for the determination of clavulanic acid in urine and plasma down to 0.1 μg/ml. This paper describes the alkaline degradation of sulbactam in methanol and in aqueous solution, and presents an HPLC method with postcolumn alkaline degradation for the determination of sulbactam in urine and plasma.

Experimental

Reagents and Materials——Sodium sulbactam was supplied by Pfizer Taito Co., Ltd. (Tokyo, Japan). Tetra-*n*-butylammonium bromide (TBAB), buffer salts, and other chemicals of reagent grade were obtained from Nakarai Chemicals Co. (Kyoto, Japan). Deionized distilled water and distilled methanol were used for the preparation of the eluents for HPLC and the solutions for measurement of UV spectra.

pH Measurements——The pH values were measured with a pH meter (model F-8, Horiba Co., Kyoto).

Measurements of UV Spectra——UV spectra were measured on a UV-240 spectrophotometer (Shimadzu Co., Kyoto) using a reference solution without sodium sulbactam.

Alkaline Degradation of Sulbactam——1) Alkaline Degradation in Methanol: i) A 1 ml aliquot of methanolic solution of sodium sulbactam (40 μg/ml) was mixed with 3 ml of NaOH-saturated methanol. After standing at room temperature for 5 min, the solution was mixed with 4 ml of 0.5 N aqueous NaOH solution. The UV spectra between 320 nm and 220 nm of the solution were measured at reaction times of 0, 20, 60, 90, and 120 min (Fig. 2). ii) A 2 ml aliquot of methanolic sodium sulbactam solution (20 μg/ml) was reacted with 2 ml of 0.5 N methanolic NaOH solution for 5 min. The UV spectrum of the solution was measured (Fig. 3a). A 2 ml aliquot of the solution was

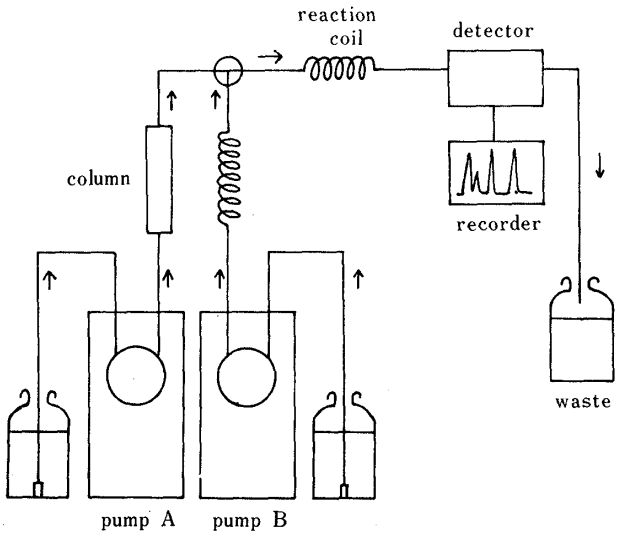


Fig. 1. Schematic Diagram of Instrumentation Used for HPLC Determination of Sulbactum

Pump A: pump for delivering eluent. Pump B: pump for delivering NaOH solution. Column: Develosil ODS-10 (25 cm × 4.6 mm i.d.). Reaction coil: 2 m × 0.25 mm i.d. Detection: 276 nm.

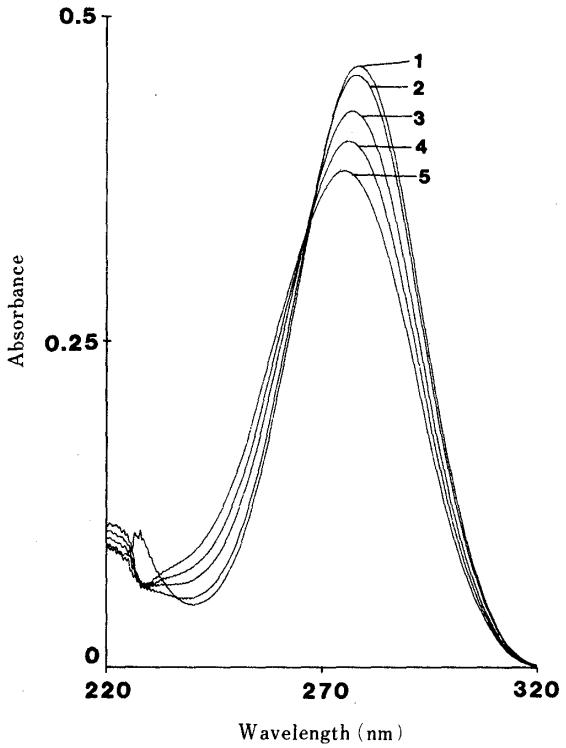


Fig. 2. UV Absorption Spectra in Aqueous NaOH Solution of Sulbactam Pretreated with NaOH-Saturated Methanol

Reaction time: 1, 0 min (immediately after addition of NaOH solution); 2, 20 min; 3, 60 min; 4, 90 min; 5, 120 min. Sample preparations; see "Experimental".

acidified with 2 ml of 1 N aqueous HCl solution (final pH 0.7), and the UV spectrum was measured (Fig. 3b). A 2 ml aliquot of the acidic solution was realkalized with 2 ml of 1 N aqueous NaOH solution (final pH 12.9), and the UV spectrum was measured (Fig. 3c).

2) Alkaline Degradation in HPLC Eluent Solutions: Sodium sulbactam was dissolved in 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution to make a final concentration of 15.0 μg/ml. A 3 ml aliquot of the solution was mixed with 1 ml of acetonitrile or methanol. Each sulbactam solution was mixed with 2 ml of 0.5 N aqueous NaOH solution. The UV spectra of the alkaline solution containing acetonitrile were measured at 0, 5, 10, 20, 40, and 80 min after addition of NaOH solution (Fig. 4a), and those containing methanol were recorded at 0, 20, 60, 90, and 120 min (Fig. 4b).

Chromatography——Figure 1 shows a schematic diagram of the instrumentation used for the postcolumn alkaline degradation of sulbactam. Pump A (TRIROTAR III, Jasco, Tokyo, Japan) was used for delivering the eluent, and pump B (TWINKLE, Jasco) for delivering aqueous NaOH solution to the reaction coil (2 m × 0.25 mm i.d.). The column used was Develosil ODS-10 (25 cm × 4.6 mm i.d., Nomura Chemicals Co., Seto, Japan) packed in stainless steel tubing. A pre-column (5 cm × 4.6 mm i.d.) packed with LiChrosorb RP-2 (E. Merck, Darmstadt, West Germany) was used to guard the main column. The eluent used was 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/methanol = 2/1 (v/v) for plasma samples, and 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/methanol = 3/1 (v/v) for urine samples. The flow rate of the eluent was maintained at 1.2 ml/min. An aqueous 0.5 N NaOH solution was delivered for the postcolumn reaction at a flow rate of 0.6 ml/min. Detection was performed at 276 nm using a UV monitor (UVIDEC-100-III, Jasco). All chromatographic operations were carried out at ambient temperature.

Preparations of Urine and Plasma Samples——A urine sample spiked with a known amount of sodium sulbactam was passed through a 0.45 μm pore size membrane filter (Fuji Photo Film Co., Tokyo, Japan). An appropriate volume of the filtrate was injected accurately through a variable loop injector (model VL-614, Jasco). A plasma sample spiked with a known amount of sodium sulbactam was mixed with 2 volumes of acetonitrile, and shaken vigorously in a Vortex-type mixer for 30 s followed by centrifugation at 3000 rpm for 5 min. An appropriate volume of supernatant was accurately injected into the chromatograph.

Results and Discussion

Alkaline Degradation of Sulbactam

Sulbactam has no UV absorption maximum above 200 nm. Figure 2 shows the changes

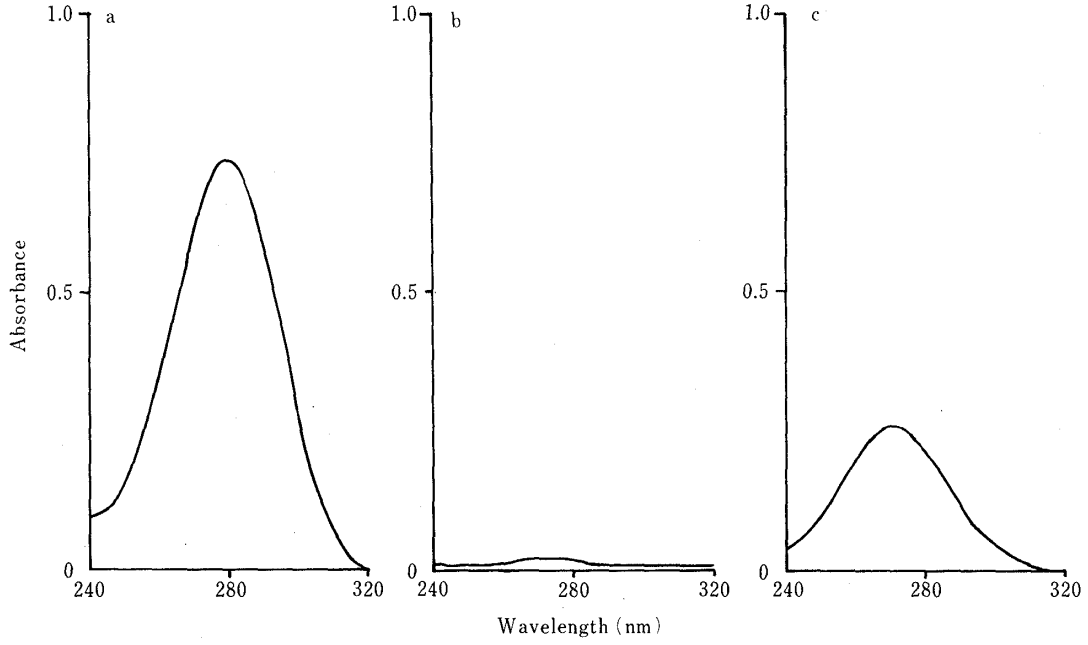


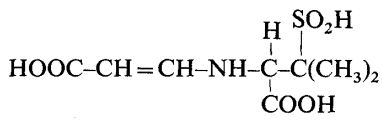
Fig. 3. pH-Dependent Spectral Change of Sulbactam after Degradation in Alkaline Methanol

Sulbactam (10 μg/ml) was degraded in 0.5 N methanolic NaOH solution for 5 min at room temperature (a). The solution was acidified with an equal volume of 1 N aqueous HCl solution (b). The acidic solution was alkalized with an equal volume of 1 N aqueous NaOH solution (c).

of UV spectra of sodium sulbactam after degradation in NaOH-saturated methanol for 5 min followed by degradation in aqueous NaOH solution. The degradation in NaOH-saturated methanol yielded a product(s) with λ_{max} 278 nm, while further degradation in aqueous NaOH solution resulted in a hypsochromic shift of λ_{max} with a concomitant decrease in the intensity at λ_{max} as the reaction proceeded. The product of λ_{max} 278 nm, which was formed immediately after addition of NaOH-saturated methanol, was stable for a few hours in NaOH-saturated methanol. Alkaline degradation of sodium sulbactam for 10 min in methanolic NaOH solution (Fig. 3a) followed by acidification with HCl to pH 0.7 resulted in a marked decrease in UV absorption (Fig. 3b). The realkalization of the solution of Fig. 3b produced a UV absorption maximum at 271 nm (Fig. 3c). However, when the realkalization was carried out after keeping the solution acidic (pH 0.7) for a longer time (60 min), the λ_{max} value of the realkalized solution changed from 271 nm to 265 nm. These results show that the product of λ_{max} 278 nm is further degraded to a substance with λ_{max} around 265 nm.

Sodium sulbactam was also degraded in aqueous alkaline solution (pH 13.4) to yield a product(s) having a UV absorption maximum at 267 nm, which was further transformed to a product(s) having a UV absorption around 260 nm.

Kemal and Knowles[13] reported that 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoic acid (II), which shows λ_{max} at 267 nm, might be formed from sulbactam enzymatically with



II

TEM-2 β-lactamase from *Escherichia coli*, and nonenzymatically at a high pH. In the previous paper,[12] we reported that potassium clavulanate was degraded in NaOH-saturated methanol to yield methyl 4-(2-hydroxyethyl)pyrrole-3-carboxylate *via* methyl 8-hydroxy-6-oxo-4-aza-2-octenoate, and in aqueous alkaline solution to yield 8-hydroxy-6-oxo-4-aza-2-octenoic acid. These results suggest that the degradation product of sulbactam having λ_{max} 278 nm may be assigned as methyl 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate (monomethyl ester of II). The results of a structural study on the alkaline degradation products of sulbactam will be reported elsewhere.

HPLC Determination of Sulbactam Using Postcolumn Alkaline Degradation

In applying the above-mentioned alkaline degradation to a postcolumn HPLC detection system, it was necessary to investigate several factors affecting the detection and separation of sulbactam. Since sulbactam was well separated from ordinary components of urine and plasma by using TBAB as an ion-pairing agent, we first examined the effects of organic modifiers (methanol and acetonitrile) and the salts added to the mobile phase on the postcolumn reaction of sulbactam. Figure 4a shows the UV absorption spectra of sulbactam in 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/acetonitrile = 3/1 (v/v) at 0, 5, 10, 20, 40, and 80 min after addition of aqueous NaOH solution (final pH 13.1). The maximum UV absorbance at 266 nm was attained at 5 min after addition of NaOH solution. Figure 4b shows the UV spectra of sodium sulbactam in 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/methanol = 3/1 (v/v) mixed with aqueous NaOH solution (final pH 13.2), recorded at 0, 20, 60, 90, and 120 min after addition. The maximum UV absorbance at 276 nm was obtained just after addition of NaOH solution (0 min). No spectral change was observed during 0—10 min. This finding was similar to that in the degradation in NaOH-saturated methanol followed by degradation in aqueous NaOH solution (Fig. 2). The results in Fig. 4 show that methanol provides a faster and higher UV response than acetonitrile, and that the alkaline degradation product(s) in methanol was more stable than that in acetonitrile. Thus, methanol was used as the organic modifier of the eluent for the present

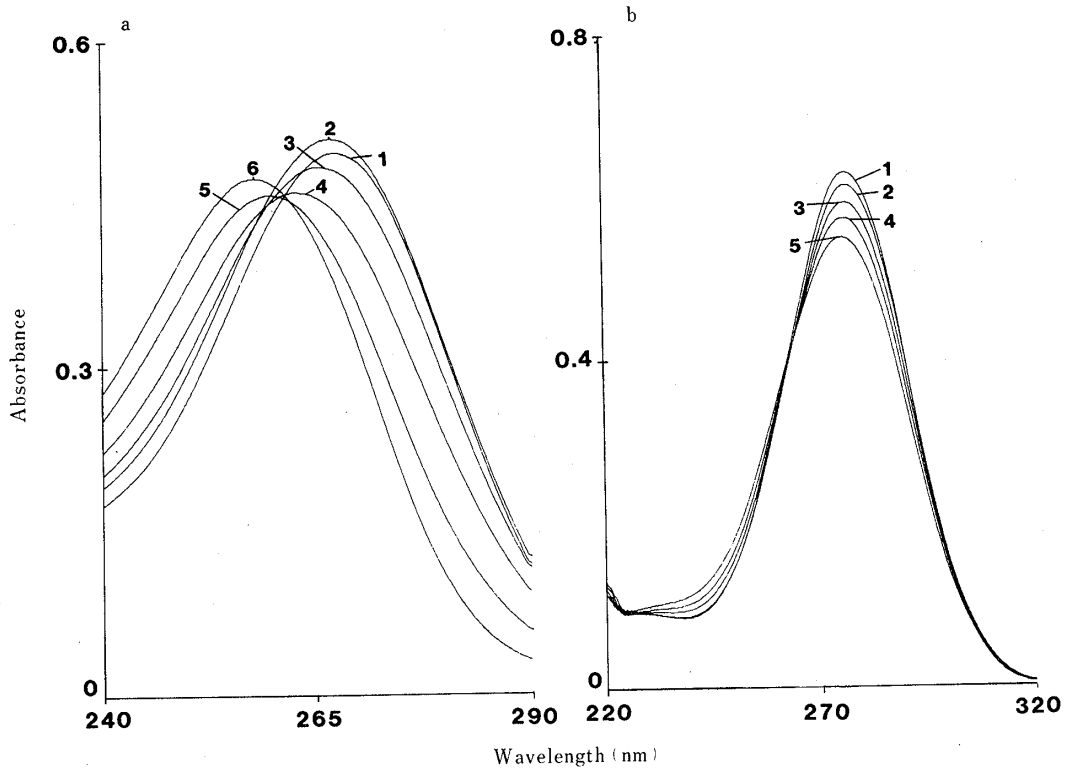


Fig. 4. UV Absorption Spectra of Sulbactam Dissolved in Alkaline Mobile Phase

Solvent: a, 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/CH_3CN = 3/1 (v/v); b, 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/MeOH 3/1 (v/v). A 2 ml aliquot of sulbactam solution (11.3 μg/ml) was mixed with 1 ml of 0.5 N aqueous NaOH solution. Reaction time. a: 1, 0 min; 2, 5 min; 3, 10 min; 4, 20 min; 5, 40 min; 6, 80 min. b: 1, 0 min; 2, 20 min; 3, 60 min; 4, 90 min; 5, 120 min.

purpose. The effects of TBAB and phosphate buffer concentrations and the organic modifier content (from 20 to 40%) on the alkaline degradation were not significant. Thus, the following eluent system was selected for HPLC analysis of sulbactam; 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/methanol = 3/1 (v/v) for urine samples, and 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4 solution/methanol = 2/1 (v/v) for plasma samples.

Next, the effects of flow rate and concentration of aqueous NaOH solution were examined in the actual detection system, where the flow rate of the eluent was fixed at 1.2 ml/min. When 0.5 N aqueous NaOH solution was delivered at a flow rate of 0.6 ml/min, the UV response at 276 nm reached the maximum. The effect of reaction time was examined by delivering 0.5 N NaOH solution at a flow rate of 0.6 ml/min to reaction coils of various lengths between 0.5 and 4 m (0.25 mm i.d.). Although a constant peak height was obtained regardless of the coil length tested, it was advantageous to use a rather long coil to reduce the noise level. The actual reaction time was less than 5 s, when a 2 m coil was used. Although a complicated degradation reaction may occur in the eluent system described above, the methanolysis product seems to be responsible for the UV absorption at 276 nm.

Under the optimum conditions thus established, a 10 μl portion of control urine spiked with sodium sulbactam (25.0 μg/ml) was injected through the variable loop injector. Figure 5a shows the resultant chromatogram, indicating the separation of sulbactam from ordinary urinary components. Figure 5b shows the separation of sulbactam (0.53 μg/ml) from ordinary plasma components. The intensities and retention times of background peaks due to urine and plasma components were not affected by addition of NaOH solution for the postcolumn reaction. The lower limit of accurate determination was as low as 1.0 μg/ml for urine samples

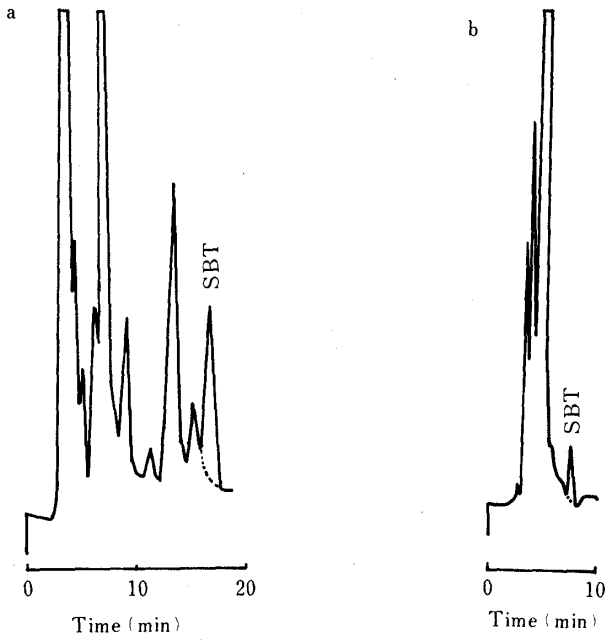


Fig. 5. Separation of Sulbactam from Ordinary Urinary (a) and Plasma (b) Components

Concentration of sulbactam: a, 25.0 μg/ml; b, 0.53 μg/ml. Injection volume: a, 10 μl; b, 60 μl. Sensitivity: a, 0.04 aufs; b, 0.01 aufs. The dotted line indicates urinary or plasma blank. SBT: sulbactam.

TABLE I. Recovery of Sulbactam from Urine and Plasma

	Added (μg/ml)	Intra-assay		Inter-assay	
		Recovery (%)	c.v. (%)	Recovery (%)	c.v. (%)
Urine	260	100	1.39	99.9	1.73
	26.0	97.3	1.17	97.2	1.28
Plasma	5.32	102	0.568	103	0.838
	0.53	93.1	1.97	94.3	3.53

($n=4$)

and 0.2 μg/ml for plasma samples. The calibration plots (peak height *vs.* concentration) were linear over the ranges of 0.2—20 μg/ml and 1—500 μg/ml for plasma and urine samples, respectively, and passed through the origin when extrapolated. Table I shows the recoveries of sulbactam from spiked urine and plasma, and the coefficients of variation. The recoveries and precision were of the order of 93.1—103% and 0.568—3.53%, respectively. This established HPLC method, described above, requires no extraction and derivatization procedures, unlike the previously reported chemical assay methods. Thus, the method is simple, rapid, and reproducible for the determination of sulbactam in urine and plasma.

It was reported that the plasma and urinary concentrations of sulbactam were 1.3 μg/ml and 299 μg/ml, respectively, at 6 h after combined intravenous administration of sulbactam (1 g) and cefoperazone (1 g).[14] Therefore, the present method should be suitable for studies on the pharmacokinetic behavior of sulbactam after administration.

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