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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF SULBACTAM USING PRE-COLUMN REACTION WITH 1,2,4-TRIAZOLE

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the determination of sulbactam in human and rat plasma and urine has been developed. Sulbactam was reacted with 1,2,4-triazole to yield a product having an ultraviolet absorption maximum at 326 nm. The product was separated using reversed-phase HPLC from the regular components of plasma and urine with an ion-pair buffer at 50°C and detected at the ultraviolet maximum. The limits of accurate determination were 0.2 and 1.0 μg/ml in plasma and urine, respectively. The coefficients of variation of inter- and intra-assays in human plasma spiked at 4.0 μg/ml (n = 5) were 1.02 and 3.05%, respectively. Coexisting cefoperazone, penicillins, or the alkaline degradation product(s) of sulbactam did not interfere in the sulbactam assay. The pharmacokinetic behaviour of sulbactam and cefoperazone coadministered to rats was estimated by moment analysis.

INTRODUCTION

Sulbactam, (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 semisynthetic β-lactamase inhibitor [1]. Microbiological assay [2] has mainly been used for the routine assay of sulbactam. Rogers et al. [3] reported a high-performance liquid chromatographic (HPLC) method for the determination of sulbactam in human plasma,

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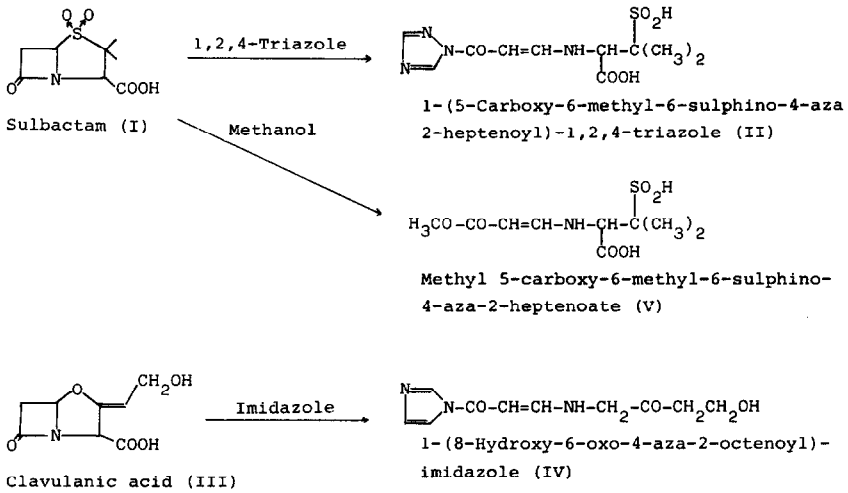

Fig. 1. Chemical structures of sulbactam, clavulanic acid and their degradation products.

saliva and urine. However, their method is not suitable for the routine monitoring of plasma or urine sulbactam levels because it involves a tedious extraction procedure and requires a long analysis time.

In a previous paper [4] we reported that sulbactam reacts with 1,2,4-triazole to yield 1-(5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoyl)-1,2,4-triazole (II) (Fig. 1), which shows an ultraviolet (UV) absorption maximum at 326 nm, and that the reaction can be used for the determination of sulbactam in pharmaceutical preparations.

This paper describes an HPLC method utilizing the above reaction for pre-column derivatization for the determination of sulbactam in plasma and urine. From the time course data obtained, the pharmacokinetic behaviour of sulbactam and cefoperazone intravenously coadministered to the rat is discussed.

EXPERIMENTAL

Reagents and materials

Sulbactam and cefoperazone were supplied by Pfizer Taito (Tokyo, Japan) and Toyama Chemical (Tokyo, Japan), respectively. 1,2,4-Triazole and tetrabutylammonium bromide (TBAB) were purchased from Nakarai Chemicals (Kyoto, Japan) and were used without further purification. Other chemicals of analytical-reagent grade were used.

A 2 *M* 1,2,4-triazole solution of pH 10.0 was prepared by dissolving 13.81 g of 1,2,4-triazole in 60 ml of distilled water, adjusting the pH to 10.0 ± 0.05 by addition of saturated sodium hydroxide solution and diluting to 100 ml with distilled water.

HPLC equipment and operating conditions

For sulbactam assay, a liquid chromatograph (Trirotar-V, Japan Spectroscopic, Tokyo, Japan) equipped with a variable-wavelength detector

(Uvidec-100-V, Japan Spectroscopic) with an 8-μl flow-through cell was used. A reversed-phase column (15 cm × 4.6 mm I.D.) packed with Develosil ODS-5 (5 μm) (Nomura Chemicals, Seto, Japan) was used with a pre-column (3 cm × 4.6 mm I.D.) packed with Develosil ODS-10 (10 μm). The pre-column was frequently repacked to guard the main column. The eluent was 5 m*M* TBAB + 1 m*M* disodium hydrogen phosphate + 1 m*M* sodium dihydrogen phosphate solution—acetonitrile (3:1) for both plasma and urine samples at a flow-rate of 1.0 ml/min. The detection wavelength was 326 nm. All chromatographic separations were carried out at 50°C.

For cefoperazone assay, a liquid chromatograph (LC-5A, Shimadzu, Kyoto, Japan) equipped with a variable-wavelength detector (SPD-2A, Shimadzu) was used with a stationary phase of Develosil ODS-10 packed in a 25 cm × 4.6 mm I.D. stainless-steel tube. The pre-column (3 cm × 4.6 mm I.D.) packed with the same material was used. The eluent was 5 m*M* disodium hydrogen phosphate + 5 m*M* sodium dihydrogen phosphate solution—methanol (2:1) at a flow-rate of 1.2 ml/min. The detection wavelength was 265 nm. All chromatographic operations were carried out at ambient temperature.

Derivatization procedure for sulbactam in plasma and urine

For plasma samples, a 50-μl aliquot of plasma was mixed with 150 μl of acetonitrile and shaken vigorously on a vortex-type mixer for 30 sec. The mixture was incubated at room temperature for 5 min. After centrifugation at 1500 *g* for 10 min, a 100-μl aliquot of the supernatant was reacted with an equal volume of the 2 *M* 1,2,4-triazole solution at 50°C for 15 min. After cooling to room temperature, the reaction solution was centrifuged at 1500 *g* for 5 min. A 20-μl aliquot of the supernatant was subjected to chromatography under the conditions described above.

For urine samples, a 100-μl portion of a neat urine was diluted ten-fold with distilled water and a 100-μl aliquot of the diluted urine was treated as described for plasma.

Rat experiments

Three male Wistar rats (285—295 g) were used for the experiments. Under pentobarbitone anaesthesia, sulbactam and cefoperazone (25 mg/kg each) dissolved in 0.9% sodium chloride solution were rapidly injected into the femoral vein. A blood sample (0.3 ml) was collected from the canulated jugular vein at 0, 5, 10, 20, 30, 40 and 60 min with a heparinized syringe. The plasma layer was obtained by centrifugation of the blood at 1500 *g* for 10 min, and stored at −20°C until assay.

For sulbactam assay, the plasma sample was treated according to the procedures described above. The standard solutions were prepared by dissolving known amounts of sulbactam in the control rat plasma to give five different concentrations (1—50 μg/ml) and were treated using the same procedures as for the plasma sample. A calibration graph of peak height versus concentration was constructed.

For cefoperazone assay, the plasma was treated as described for sulbactam, with the exceptions that methanol was used as protein precipitant and no derivatization was required. The five cefoperazone standards in plasma (1—50

μg/ml) were treated according to the procedures described above. A calibration graph of peak height versus concentration was constructed.

Pharmacokinetics

The area under the plasma concentration—time curve (AUC) and the mean residence time (MRT) in the systemic circulation are defined as

$$\mathrm{AUC} = \int_0^\infty C_p dt \quad (1)$$

$$\mathrm{MRT} = \int_0^\infty tC_p dt / \int_0^\infty Cp dt \quad (2)$$

where C_p is plasma concentration—time course [5]. AUC and MRT were calculated by linear trapezoidal integration with extrapolation to infinite time. The total body clearance (Cl_T) and steady-state volume of distribution (V_{ss}) [6] are given by

$$Cl_T = D/\mathrm{AUC} \quad (3)$$

$$V_{ss} = Cl_T\,\mathrm{MRT} \quad (4)$$

where D is a rapid intravenous dose.

RESULTS

HPLC conditions

It has been shown that the reaction of sulbactam with 1,2,4-triazole yields a product having strong UV absorption at 326 nm [4]. We tried to apply the reaction to the determination of sulbactam in human and rat plasma and urine. The reaction product (II) was separated from the regular components of plasma and urine on an ion-pair reversed-phase HPLC column. When the mobile phase did not contain an ion-pairing agent (TBAB), the retention time of II was markedly shortened, resulting in a poor separation from background peaks. The separation was also improved by elevating the column temperature to 50°C, at which II eluted as a single, sharp peak.

Reaction conditions

The effects of the concentration and pH of 1,2,4-triazole solution and the reaction temperature on the formation of II in human plasma and urine samples were examined in order to establish a routine assay procedure for sulbactam. The sample solution was reacted with an equal volume of the reagent solution, and a 20-μl portion was accurately removed at appropriate reaction times and subjected to the HPLC analysis. The reaction of sulbactam with 1,2,4-triazole in pooled human plasma (not deproteinized) followed by the precipitation of plasma proteins with methanol or acetonitrile gave no peak corresponding to II on the chromatogram. Also, the precipitation with methanol followed by the reaction with 1,2,4-triazole gave a lower response than the precipitation with acetonitrile. Therefore, sulbactam was recovered with three volumes of acetonitrile and reacted with 1,2,4-triazole. When a 2 *M* 1,2,4-triazole solution of pH 10.0 was used at a reaction temperature of 50°C, a maximum and constant peak height was obtained at 15 min. Hence the

conditions previously described [4] were selected for the routine assay of sulbactam in human plasma.

Corresponding results were obtained with urine samples, leading to the same reaction conditions. The reaction of sulbactam with the 2 *M* 1,2,4-triazole solution in neat urine gave a peak height only about 60% of that in distilled water. Therefore, the urine sample was diluted ten-fold.

The reaction conditions employed for the assay of sulbactam in rat plasma and urine were the same as those used for human plasma and urine.

HPLC analysis

Under the HPLC and reaction conditions established above, a 20-μl portion of a treated human plasma or urine sample was subjected to chromatography. Figs. 2 and 3 show that the derivatized sulbactam (II) was completely separated from the regular components of human plasma and urine within 8 min after injection. The chromatograms obtained from the treated rat plasma and urine samples were almost identical with those from human plasma and urine. The

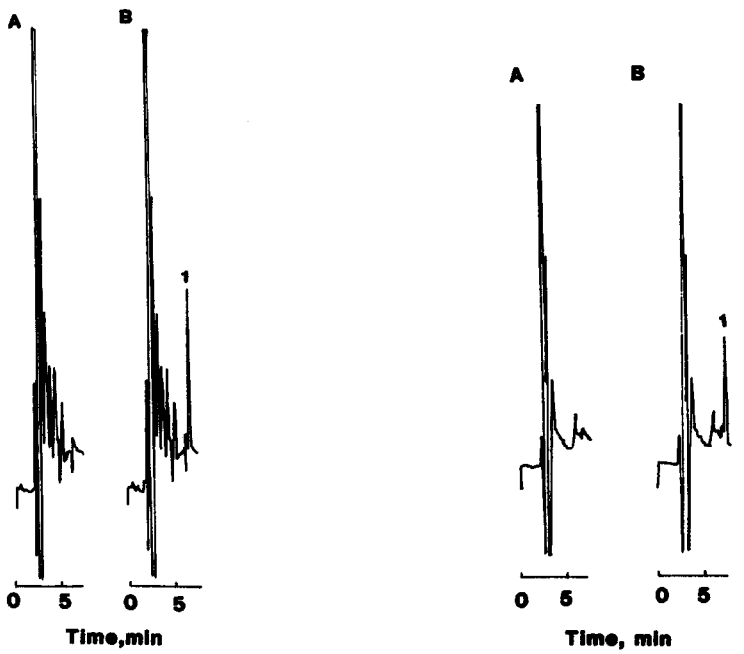

Fig. 2. Chromatogram of derivatized sulbactam in human plasma. The eluent was monitored at 326 nm and 0.04 a.u.f.s. Control plasma (A) and control plasma spiked with sulbactam (1.0 μg/ml) (B) were treated according to the derivatization procedures described under Experimental. A 20-μl portion of each sample solution was subjected to chromatography. Peak 1 is 1-(5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoyl)-1,2,4-triazole.

Fig. 3. Chromatogram of derivatized sulbactam in human urine. The eluent was monitored at 326 nm and 0.08 a.u.f.s. Control urine (A) and control urine spiked with sulbactam (5.0 μg/ml) (B) were treated according to the derivatization procedures. A 20-μl portion of each sample solution was subjected to chromatography. Peak 1 is 1-(5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoyl)-1,2,4-triazole.

lower limit of accurate determination was as low as 0.2 μg/ml for human plasma samples and 1.0 μg/ml for human urine samples. The calibration graphs for sulbactam with the concentrations ranging from 0.2 to 8 μg/ml for human plasma samples and from 5 to 80 μg/ml for human urine samples were linear and passed through the origin, with correlation coefficients of 0.999 and 0.998, respectively.

Recovery and interference

Table I shows the recoveries of sulbactam from spiked human plasma and urine, and the coefficients of variation.

The interferences of penicillins (ampicillin, amoxicillin, and penicillin G), cefoperazone and the alkaline degradation product(s) of sulbactam were examined. Sulbactam (5 μg/ml) and penicillins or cefoperazone (each at a concentration of 500 μg/ml) were reacted with 1,2,4-triazole under the assay con-

TABLE I

RECOVERY OF SULBACTAM FROM URINE AND PLASMA

Average and coefficient of variation (C.V.) for five analyses.

Sample	Added (μg/ml)	Inter-assay		Intra-assay	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Urine	40.0	100.6	0.90	99.9	1.73
Plasma	4.0	100.5	1.02	105.8	3.50

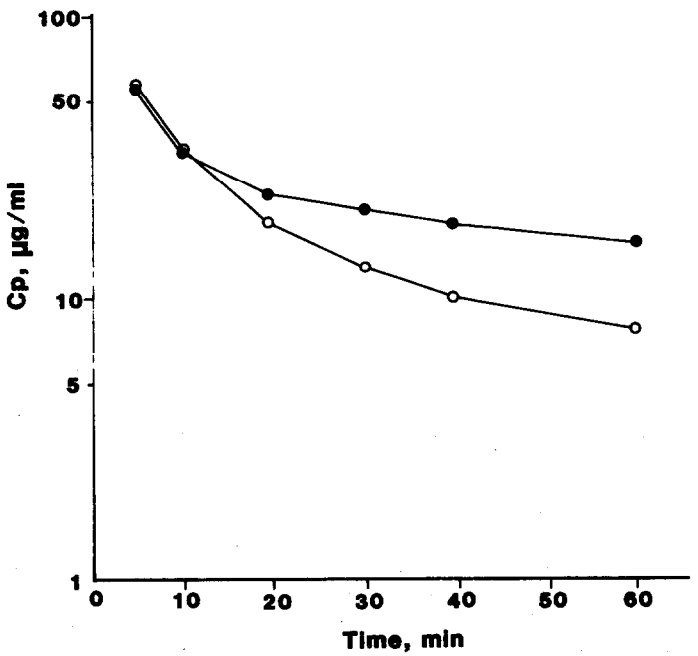

Fig. 4. Mean semi-logarithmic plots of plasma concentrations (C_p) of sulbactam (•) and cefoperazone (○) coadministered to three male rats.

ditions. There was no influence on the sulbactam quantitation in any instance. The alkaline degradation product(s) of sulbactam (1 mg/ml) (0.5 *M* sodium hydroxide solution for 10 min followed by neutralization) did not interfere in the sulbactam (5 μg/ml) assay.

Pharmacokinetic studies

Fig. 4 shows semi-logarithmic plots of the time courses following an intravenous combined dose of sulbactam and cefoperazone to rats. Table II shows the disposition properties of sulbactam and cefoperazone estimated by the non-compartmental method.

TABLE II

IN VIVO CHARACTERISTICS OF SULBACTAM AND CEFOPERAZONE IN RATS AFTER AN INTRAVENOUS COMBINED DOSE OF SULBACTAM AND CEFOPERAZONE

Data are given as mean values ± standard deviations (S.D.) for three rats.

Parameter	Sulbactam		Cefoperazone	
	Mean	S.D.	Mean	S.D.
AUC (mg min ml^{-1})	2.11	0.19	1.26	0.34
MRT (min)	56.9	8.4	29.5	3.4
V_{ss} (ml kg^{-1})	675	81	602	112
Cl_T (ml min^{-1} kg^{-1})	11.9	1.0	20.8	6.2

DISCUSSION

It has been reported that the reaction of clavulanic acid (III) (Fig. 1), which is a β-lactamase inhibitor isolated from *Streptomyces clavuligerus* ATCC 27064, with imidazole yields 1-(8-hydroxy-6-oxo-4-aza-2-octenoyl)imidazole (IV), having a UV absorption maximum at 312 nm [7]. Foulstone and Reading [8] applied the method to the HPLC determination of clavulanic acid in human plasma and urine. Bird et al. [7] reported that the *cis* and *trans* isomers of IV coeluted on a reversed-phase HPLC column. The same effect may be responsible for the broad peak with a shoulder that was observed when II was chromatographed in an ion-pairing mode at room temperature. For the purpose of HPLC quantitation, however, it is preferable that the isomers coelute as a single peak. This was achieved by elevating the column temperature to 50°C.

It has been found that sodium sulbactam was degraded to methyl 5-carboxy-6-methyl-6-sulphino-4-aza-2-heptenoate (V) in methanolic solution [9]. The recovery of sulbactam bound to plasma proteins with methanol followed by reaction with 1,2,4-triazole at 50°C might yield V. Therefore, the precipitation with methanol is unsuitable for this assay purpose.

It has been reported [10, 11] that the elimination of sulbactam from man and dog is slightly more rapid than that of cefoperazone, and that the coadministration of sulbactam and cefoperazone has no mutual influence on the pharmacokinetic features in man. However, as shown in Table II, the MRT of sulbactam in the rat is approximately double that of cefoperazone, that is,

sulbactam is eliminated more slowly than cefoperazone from the rat. As the V_{ss} value of sulbactam is almost the same as that of cefoperazone, the large difference in MRT values between sulbactam and cefoperazone is due to the differences in their Cl_T values. This coincides well with the previous result [12] that cefoperazone was eliminated rapidly from the rat but eliminated slowly from other species (man, rabbit, dog and monkey). Detailed consideration of the pharmacokinetic features of sulbactam and cefoperazone will be given elsewhere.

The proposed HPLC assay method for sulbactam in plasma and urine is specific to intact sulbactam without interferences from penicillins, cefoperazone and the alkaline degradation product(s) of sulbactam. It requires only simple treatment procedures and the elution time is as short as 8 min. Therefore, this method will be useful for the determination of sulbactam coadministered with penicillins or cefoperazone in clinical samples.

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