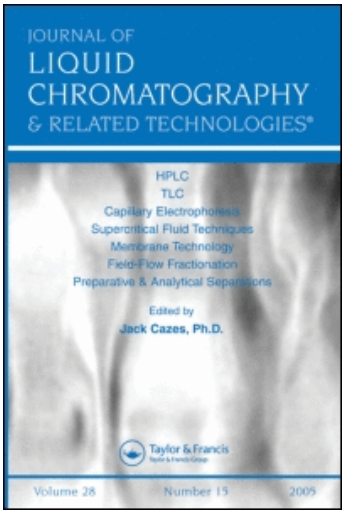

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ABSTRACT

A high performance liquid chromatography (HPLC) method has been developed for the separation and quantitation of clavulanic acid (CA), sulbactam (SUL), and tazobactam (TAZ) in human sera. The optimum mobile phase used in conjunction with a *β*-cyclodextrin (*β*-CyD) column contained methanol-5 mM tetraethylammonium acetate (TEAA) (35 : 65, v/v, pH 4.5) and 1.0 mL/min flow rate for chromatographic separation. The mean recoveries of CA, SUL, and TAZ range from 84.4 to 87.8%, 88.4 to 90.6%, and 89.0 to 90.8% respectively, from human serum samples spiked with the inhibitors, are routinely obtained. The lower limit of quantitation (<0.156 μg/mL) and the low

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coefficient of variation (<3.92%) assure that this method is sensitive and reproducible.

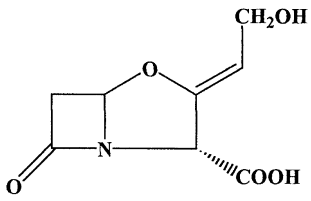
Key Words: β-Lactamase inhibitors; HPLC; β-Cyclodextrin; Serum sample

INTRODUCTION

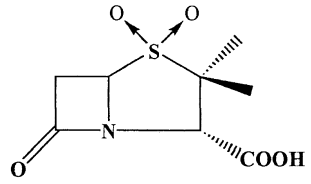
Infection caused by β-lactamase producing bacterial strains has recently become a major problem in hospitals. Several β-lactamase inhibitors have been developed against the target enzyme. Clavulanic acid (CA), produced by *Streptomyces clavuligerus*, is a potent β-lactamase inhibitor,[1] while sulbactam (SUL) and tazobactam (TAZ) are the semi synthetic products.[2–4] When they are combined with some penicillins or cephalosporins, the mixed ingredients have been shown effective against various β-lactamase producing bacteria both in vitro and in vivo.[5–12] Amoxicilin-CA, ampicillin-SUL, and piperacillin-TAZ are currently undergoing clinical trials for the treatment of severe β-lactamase-producing bacteria infections.[13]

Several methods have been described for the determination of the β-lactamase inhibitors in biological fluids, such as microbiological assay,[9–14] isotachophoresis,[15,16] and flow-injection chemiluminometry,[17] though these methods are generally not specific. Improved high performance liquid chromatography (HPLC) techniques using C18 columns has also been developed for the separation and quantitation of CA and SUL in plasma, urine, or cerebrospinal fluid.[18–26] Due to interference and broadening of the separated peaks, the application of these methods is limited.

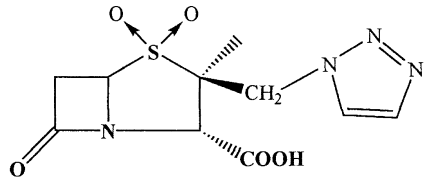
β-Cyclodextrin (β-CyD) is a macrocyclic oligosaccharide consisting of seven glucose units. It forms a toroidal structure with a hydrophilic exterior face and a hydrophobic inner cavity. Armstrong developed the first high efficiency bonded β-CyD phase on 5 μm silica gels as a packing material for the liquid chromatographic separation of optical, geometrical, and structural isomers.[27–29] Since then, many researchers have reported the separation and determination of drugs simultaneously by employing bonded β-CyD column.[30–35] Previously, we have reported the separation of a series of cephalosporins by HPLC with β-CyD stationary phase and the retention behavior of β-lactam structural compounds with relation to β-CyD cavities.[36] Molecules of CA, SUL, and TAZ contain a β-lactam ring and they are structurally similar to each other (Fig. 1). In this study, by employing a HPLC method with β-CyD stationary and mobile phases, the three β-lactamase inhibitors, CA, SUL, and TAZ, and a wide range of β-lactam antibiotics are analyzed and successful separation and quantitation of the β-lactamase inhibitors is reported.



clavulanic acid (CA)



sulbactam (SUL)



tazobactam (TAZ)

Figure 1. Structure of CA, SUL, and TAZ molecules.

EXPERIMENTAL

Materials

Lithium clavulanate, sodium sulbactam, and sodium tazobactam were obtained as USP reference standards (Rockville, MD). Ampicillin, amoxicillin, cefaloridine, and cefaperazone were purchased from Sigma (St. Louis, MO). Piperacillin (Lederle, USA) was purchased from local drug stores. The reagents such as acetonitrile, methanol, glacial acetic acid, methylene chloride, phosphate buffer, and sodium hydroxide were obtained from E. Merck (Darmstadt, Germany). Tetraethylammonium acetate (TEAA), and 3,4-dihydroxybenzoic acid (as internal standard, IS) were obtained from Aldrich (Milwaukee, WI). Distilled water was deionized twice before use by passing through a Milli-Q

reagent water system (Millipore, USA). Human blood was collected from healthy volunteers. After storing in a 4°C refrigerator for 6 h, the coagulated blood was centrifuged at 1800*g* for 15 min to obtain drug-free serum.

Methods

The liquid chromatographic system consists of a Waters Model 6000A pump connected to a U6K injector, a photodiode array detector model 990, and a model 5200 printer/plotter (Waters Associates, Milford, MA). The separation was accomplished on a 5 μm bonded *β*-CyD column (Cyclobond I, 250 mm × 4.6 mm I.D.), which was purchased from Advanced Separation Technologies Inc. (Whippany, NJ). The post column system was assembled with a high pressure pump connected to a mixing reaction chamber (Beckman, 110B solvent delivery pump and 230 post column reactor, USA). Quantitation was based on integration of peak areas using a NEC model APC IV power mate 2 computing integrator (NECIS, MA). A model 112-pH meter (Photovolt, NY) was used to measure the pH values of mobile phases. Data calculation was performed using Sigma 2000 statistics software.

Chromatographic Conditions

For the chromatographic conditions, wide ranges of mobile phase (TEAA concentration 0–12.5 mM, pH 3.5–7.0, and methanol content 27.5–50%) were studied. Tetraethylammonium acetate solutions were adjusted with glacial acetic acid until the desired buffer pH was obtained. Mobile phase was prepared by mixing methanol with buffer solution, degassed by bubbling helium about 10 min, and then filtered through a 0.45 μm filter (Millipore, Yonezawa, Japan). The flow rate was adjusted at 1.0 mL/min and 20 μL sample solutions were injected. The attenuation unit for full-scale deflection was set at 0.002–1.2 AUFS. The post column reagent was 0.3 N sodium hydroxide solution and delivered at a flow rate of 1.0 mL/min to a mixing reaction chamber. Detection was performed at UV 225 and 270 nm at ambient temperature. The capacity factor of one peak was defined by relating the retention time (t_R) of the peak to the retention time of the unretained peak (t_0); thus, $k' = (t_R - t_0)/t_0$.

Specificity

To evaluate the specificity of the method, 0.5 mL of sample solution was carried through the assay procedure and the retention times of endogenous

compounds in buffer solution were compared with the inhibitors. The interference from other antibiotics that could be co-administered was studied. The following drugs were checked: ampicillin, amoxicillin, cefaloridine, cefoperazone, and piperacillin.

Procedure for Standard Curve

Stock solutions of CA, SUL, TAZ inhibitors and the internal standard (IS) were prepared by adding 40 mg of lithium clavulanate, sodium sulbactam, sodium tazobactam, and 20 mg of 3,4-dihydroxybenzoic acid into each of 100 mL volumetric flasks and dissolving with distilled water, respectively. Serial dilution samples were obtained by mixing CA, SUL, TAZ, and IS stock solutions into drug-free serum and then diluting to give the concentrations ranging from 0.2 to 25 μg/mL for inhibitors and 6.25 μg/mL for IS. To measure the drugs in serum, each spiked sample (2 mL) was shaken by vortexing (Thermolyne, Iowa, USA) for 5 min and then adding acetonitrile (4 mL) for deproteinization. After centrifugation for 15 min at 1500*g* (Hsiangtai CN-2060, Taiwan), 2.0 mL supernatant was transferred to another tube and equal volume of methylene chloride was added for re-extraction. The aliquot was withdrawn, passed through a Centricon YM-10 filter (Millipore, MA), and then 20 μL of the filtrate was injected onto the *β*-CyD chromatographic system. The resulting peak areas were measured and calibration curves were obtained by plotting the peak area ratio of each inhibitor to IS against the drug concentration over the ranges.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The LOQ was determined from the peak and the standard deviation of the signal-to-noise ratio (S/N). The LOQ was defined as the sample concentration of these inhibitors resulting in a peak area of eight times the S/N. The LOD was defined as the sample concentration of inhibitors resulting in a peak area of three times the S/N.

Accuracy and Precision

The intra-day assay accuracy and precision of the method were evaluated in spiked serum over a high, middle, and low concentration range (20, 5.0, and 0.5 μg/mL). The inter-day assay accuracy and precision of the assay were evaluated over two days. The concentrations of the samples used were the same as those utilized for the intra-day study.

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Recovery

The extraction efficiencies (recoveries) studies were carried out by adding known amounts of IS, CA, SUL, and TAZ to drug-free serum or water matrix, respectively. The extraction processes were the same as described above. Recovery was determined as the ratio of average peak areas of the drug-free serum spiked with known amounts of drugs (20, 5.0, and 0.5 μg/mL) to the average peak areas of the water extract at the same concentrations.

RESULTS AND DISCUSSION

Effects on the Chromatography of β-Lactamase Inhibitors

In chromatographic studies, use of a β-CyD column represents a new approach for the simultaneous separation of β-lactamase inhibitors. The added TEAA in mobile phase is an important factor that affects the retention of CA, SUL, and TAZ. Figure 2 shows the retention behavior of these compounds over a wide range of TEAA concentrations from 0 to 12.5 mM with a mobile phase of MeOH–TEAA buffer 35 : 65 (v/v), pH = 4.5. It was found, that if there is no TEAA ion in the mobile phase, longer retention and worse peak shapes of the analytes were observed. In reverse, a relatively fast decreasing in retention occurs with increasing TEAA concentration. The results indicate that higher concentration of TEAA in the mobile phase part masks the absorption sites of β-CyD cavities and renders the strength of inclusion complexation between the inhibitors and the β-CyD cavities weaker.

When the pH of the same buffer system was increased, a shorter retention of these inhibitors was obtained (Fig. 3). Clavulanic acid, SUL, and TAZ have pKa values of 2.7, 2.3, and 2.1, respectively.[13] Between pH ranges of 3 and 7, these inhibitors are completely ionized and combined with TEAA ions to form ion-pair complexes, which are more retarded in the hydrophobic cavities. Thus, by increasing pH of the mobile phase of the buffer, the increased OH^- ion concentration competes with the carboxylate groups of β-lactam inhibitors for interaction with TEAA ions. As a result, a decreased retention time of inhibitors in β-CyD accompanied with the increase of pH is observed.[37]

Organic solvents also affect the retention time of these inhibitors. Figure 4 shows that when methanol concentration was increased on a methanol–water ratio in the mobile phase, a short retention time of these inhibitors was obtained. The same results were obtained with the acetonitrile–water mobile phase. This phenomenon has been interpreted in the cyclodextrin-binding studies as that an increase in organic solvent content in the mobile phase will weaken the strength

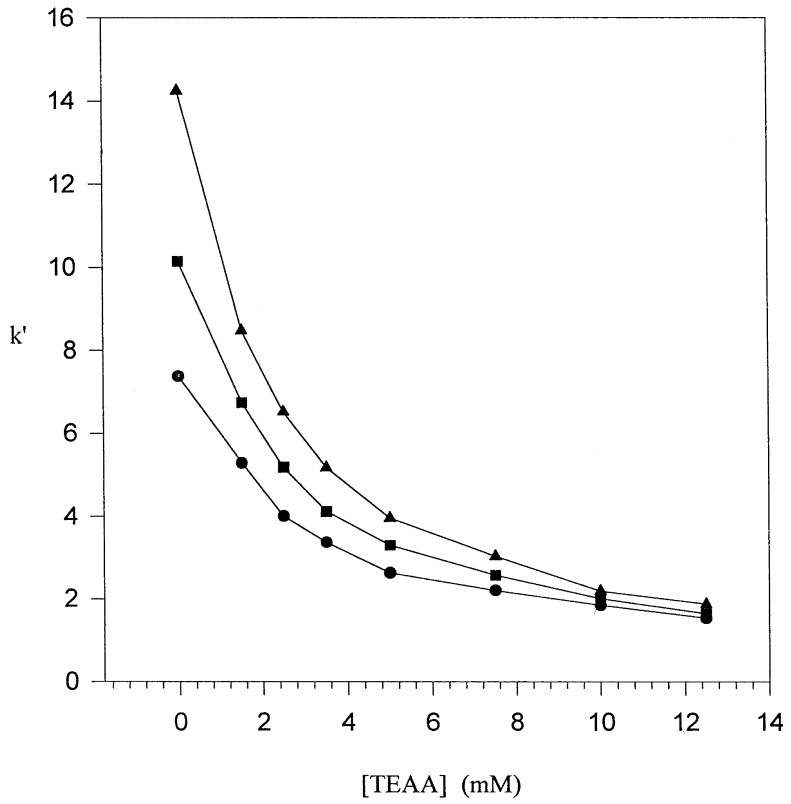

Figure 2. Effect of TEAA concentration on the retention capacity of the inhibitors. Chromatographic condition: column, Cyclobond I, 250 × 4.6 mm I.D.; mobile phase, methanol–TEAA buffer (35 : 65, v/v), pH = 4.5; flow rate, 1.0 mL/min; detection, UV 270 nm; post column, 0.3 N NaOH; flow rate, 1.0 mL/min. Labels: CA (●); SUL (■); TAZ (▲).

of inclusion complexation between guest molecules and the hydrophobic β-CyD cavities.[28,38,39]

Chromatogram of β-Lactamase Inhibitors

Chromatograms of CA, SUL, and TAZ spiked in human sera were performed. On a β-CyD bonded column, using 3,4-dihytroxybenzoic acid as IS and the mobile phase of methanol-5 mM TEAA (35 : 36, v/v, pH 4.5), a clear separation with high resolution of all participated compounds has been achieved

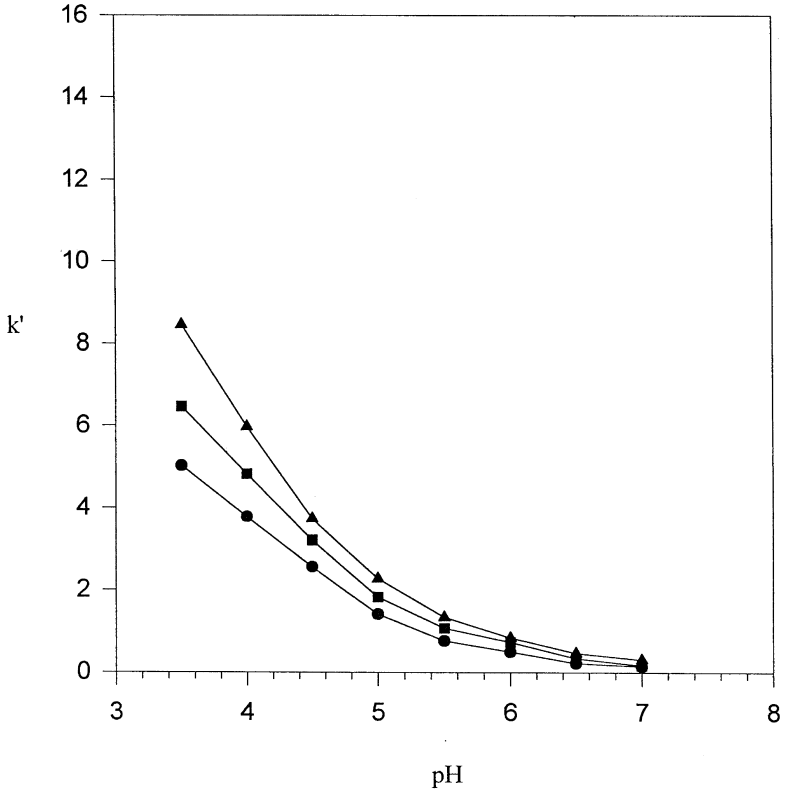

Figure 3. Effect of pH on the retention capacity of the inhibitors. Chromatographic condition: mobile phase, methanol–5 mM TEAA buffer (35 : 65, v/v). Other factors are the same as in Fig. 2. Labels: CA (●); SUL (■); TAZ (▲).

as measured at 270 nm UV. The retention time of IS, CA, SUL, and TAZ are 7.54, 10.71, 12.63, and 15.46 min, respectively. Extraction and chromatographic analyses of blank samples confirm that there are no endogenous peaks coeluting with the β-lactamase inhibitors. Addition of ampicillin, amoxicillin, cefaloridine, cefaperazone, and piperacillin in the assay system does not show any interference in the analyses of these inhibitors (Fig. 5d). Previously, Haginaka et al.[21,26] reported that a sensitive HPLC method was developed for the determination of CA and SUL in biological fluids on a C18 reversed-phase column after alkaline degradation. In this study, post-column alkaline degradation of CA, SUL, and TAZ showed 6–8 times of peak responses measured at 270 nm UV than that measured at 225 nm UV (Fig. 5b, 5c).

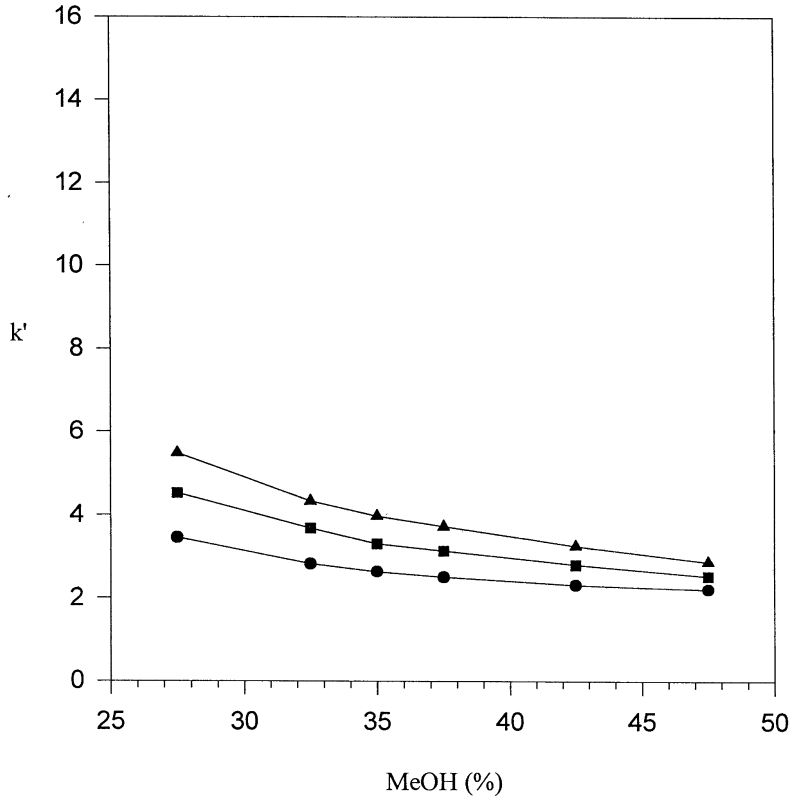

Figure 4. Effect of methanol content on the retention capacity of the inhibitors. Chromatographic conditions are the same as in Fig. 2, except the TEAA concentration is 5 mM and methanol content is ranged from 27.5–47.5%. Labels: CA (●); SUL (■); TAZ (▲).

Determination of *β*-Lactamase Inhibitors

Under the optimal operating condition, simultaneous quantification of CA, SUL, and TAZ could be achieved when the internal standard is included. The calibration curves of mixed samples are carried out in serum with eight different concentrations ranging from 0.2 to 25 μg/mL. The regression analysis between peak area ratios and serum concentrations are linear and the correlative coefficients (r) of inhibitors are better than 0.998. The lower limit of quantitation (S/N = 8) is 0.156 μg/mL for CA, SUL, and TAZ. The LOD of these inhibitors is as low as 0.05 μg/mL (S/N = 3). The intra-day and inter-day assay precisions and accuracies are characterized by relative standard deviations (RSD) and relative

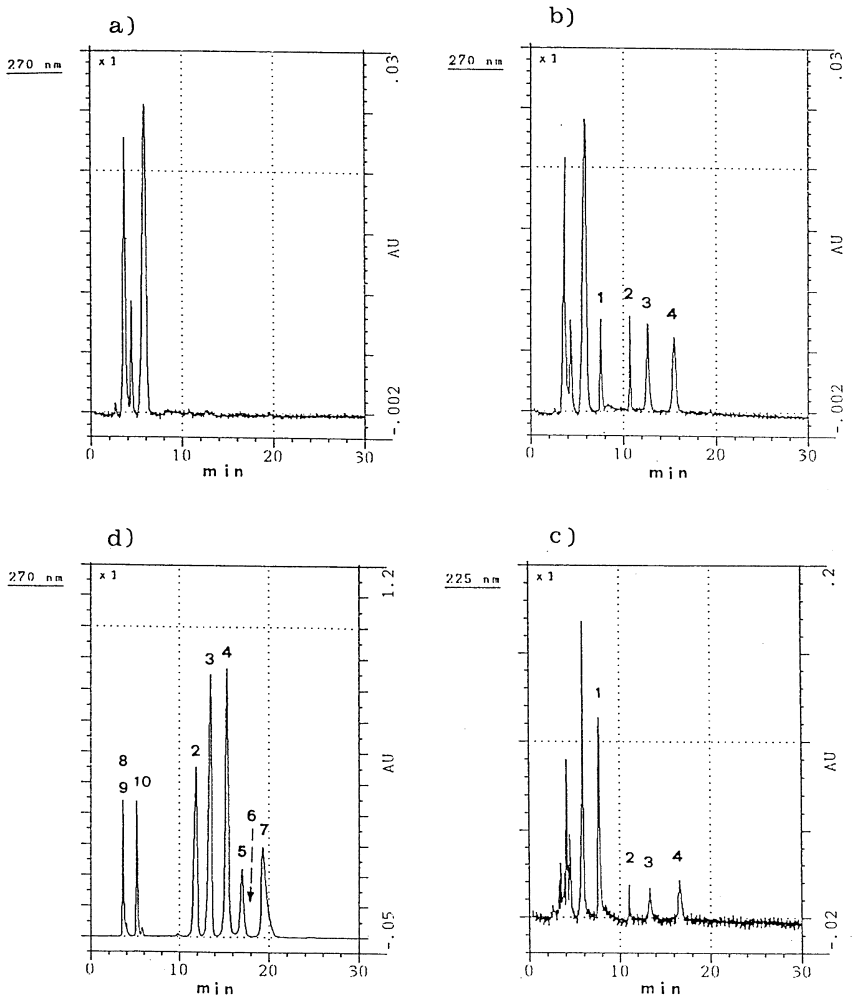

Figure 5. Chromatograms: (a) blank serum; (b) serum spiked with inhibitors (0.313 μg/mL) and IS (6.25 μg/mL), detection UV 270 nm; (c) samples as (b) except inhibitors were 2.5 μg/mL and detected at UV 225 nm; (d) water matrix spiked with inhibitors and antibiotics. Conditions are the same as in Fig. 2, except the TEAA concentration is 5 mM. Peaks: 1, IS; 2, CA; 3, SUL; 4, TAZ; 5, cefoperazone; 6, piperacillin; 7, cefazolin; 8, amoxicillin; 9, ampicillin; 10, cephaloridine.

errors (RE) for the spiked samples at high, middle, and low concentrations (20, 5, and 0.5 μg/mL). The results are shown in Table 1. Precisions for all targeted compounds are less than 6.0%. The accuracy values for CA, SUL, and TAZ are below 16%, 10%, and 15%, respectively. It means that the method is sensitive and reproducible. Recovery studies could be obtained by comparing the found concentrations of the inhibitors of drug-free serum to the data of water matrix. In serum assays, acetonitrile is preferable to methanol for the extraction procedure, because CA is decomposed gradually in the latter solvent. Table 2 shows the results of high, middle, and low concentrations of CA, SUL, and TAZ in serum sample. The mean extraction recoveries of CA range from 84.4 to 87.8%, while those data of SUL and TAZ are between 88.4 and 90.6%, respectively. The coefficient of variation is less than 2.8% for CA, 2.7% for SUL, and 2.1% for TAZ. These results indicate that the currently developed method is suitable for monitoring these inhibitors in human sera.

Table 1. Accuracy and Precision of β-Lactamase Inhibitors in Serum

	Concentration Added (μg/mL)	Concentration Found (μg/mL)	Relative Standard Deviation (%)	Relative Error (%)
		Intra-day assay ($n = 3$)		
CA	20.0	19.35 ± 0.56	2.9	−3.3
	5.0	4.83 ± 0.16	3.3	−3.4
	0.5	0.48 ± 0.02	4.2	−4.0
SUL	20.0	19.78 ± 0.42	2.1	−1.1
	5.0	4.89 ± 0.11	2.3	−2.2
	0.5	0.49 ± 0.02	3.3	−2.0
TAZ	20.0	19.51 ± 0.38	2.0	−2.5
	5.0	4.85 ± 0.13	2.7	−3.0
	0.5	0.48 ± 0.02	4.2	−4.0
		Inter-day assay ($n = 3$)		
CA	20.0	18.25 ± 0.70	3.86	−8.8
	5.0	4.62 ± 0.24	5.18	−7.6
	0.5	0.42 ± 0.03	7.22	−16.0
SUL	20.0	18.80 ± 0.77	4.09	−6.0
	5.0	4.68 ± 0.23	4.81	−6.4
	0.5	0.45 ± 0.02	4.97	−10.0
TAZ	20.0	18.53 ± 0.73	3.96	−7.4
	5.0	4.66 ± 0.21	4.53	−6.8
	0.5	0.43 ± 0.02	5.46	−14.0

Table 2. Recoveries of β-Lactamase Inhibitors in Human Serum Samples

Compound	Concentration (μg/mL)	Added Recovery[a] (%) Mean ± SD	C.V. (%)
CA	20.0	84.4 ± 2.1	2.49
SUL	20.0	90.6 ± 1.7	1.83
TAZ	20.0	90.2 ± 1.4	1.56
CA	20.0	87.8 ± 2.5	2.82
SUL	20.0	88.4 ± 1.9	2.14
TAZ	20.0	90.8 ± 1.8	1.94
CA	20.0	86.8 ± 3.4	3.92
SUL	20.0	89.5 ± 2.3	2.63
TAZ	20.0	89.0 ± 1.9	2.09

[a]R(%) = [compound in serum] × 100/[compound in aqueous solution].
Results are the mean of three replicate analyses.

CONCLUSION

Using β-CyD stationary phase, we have been able to develop a new HPLC method for the determination of β-lactamase inhibitors in human serum simultaneously. The added TEAA concentration, pH, and methanol content of the mobile phase are important factors that affect the retention time of CA, SUL, and TAZ. In serum assay, there is a clear resolution of the inhibitors using a mobile phase of methanol-5 mM TEAA (35 : 65, v/v, pH 4.5), flow rate 1.0 mL/min, and detection at UV 270 nm. The limit of quantitation (S/N = 8) is 0.156 μg/mL for CA, SUL, and TAZ. The mean extraction recovery of CA ranged from 84.4 to 87.8%, while SUL and TAZ are measured between 88.4 and 90.6%. The lower analytic quantity and the less coefficient of variation exhibit that the current method is sensitive and reproducible. Thus, it is concluded that the proposed method is a useful alternative for the determination of β-lactamase inhibitors in biological fluids.

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