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Simultaneous determination of ticarcillin and clavulanate in rabbit serum and tissue cage fluid by liquid chromatography

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Abstract

A gradient elution HPLC method with a wavelength switch technique was developed to simultaneously analyze the β-lactam ticarcillin and the β-lactamase inhibitor clavulanate in rabbit serum and tissue cage fluid (TCF). A C18 reversed-phase column with a programmable UV detector changing the wavelength from 218 to 254 nm at 9 min was used for chromatographic separation. The mobile phase consisted of acetonitrile, phosphate buffer and tetrabutylammonium hydrogen sulfate by following a gradient elution program at a flow-rate of 1 ml/min. Sample processing was carried out with liquid–liquid extraction. Good linearity, recoveries, precision and accuracy were obtained. The ranges of the standard curves were 1–100 μg/ml for ticarcillin, and 0.2–20 μg/ml for clavulanate. This assay has been successfully applied to analyze ticarcillin and clavulanate in rabbit serum and tissue cage fluid samples from a pharmacokinetic study.

Keywords: Ticarcillin; Clavulanate

1. Introduction

Ticarcillin (Fig. 1a), α-carboxy-3-thienylmethylpenicillin, is a semi-synthetic α-carboxypenicillin, which is commercially available and used as a mixture of epimers (*R*- and *S*-type) in the clinical form. There is no stereoselectivity in the pharmacokinetics of ticarcillin in humans [1]. Ticarcillin is active against a variety of Gram-positive and Gram-negative aerobic and anaerobic bacteria, but it is susceptible to hydrolysis by a range of β-lactamases. Clavulanate (Fig. 1b), 3-(2-hydroxyethylidene)-7 - oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid, is structurally similar to penicillins and cephalosporins, the β-lactam ring in clavulanate is fused with an oxazolidine ring rather than a thiazolidine ring as in penicillins or a dihydrothiazine ring as in cephalosporins [2]. Clavulanate is an irreversible inhibitor of many bacterial β-lactamases with weak antibacterial activity when used alone. Ticarcillin in combination with clavulanate expands ticarcillin's broad antibacterial spectrum. The commercial product of the combination of ticarcillin and clavulanate (Timentin®) consists of 30 parts ticarcillin and one part clavulanate [3].

Several methods have been reported to quantify

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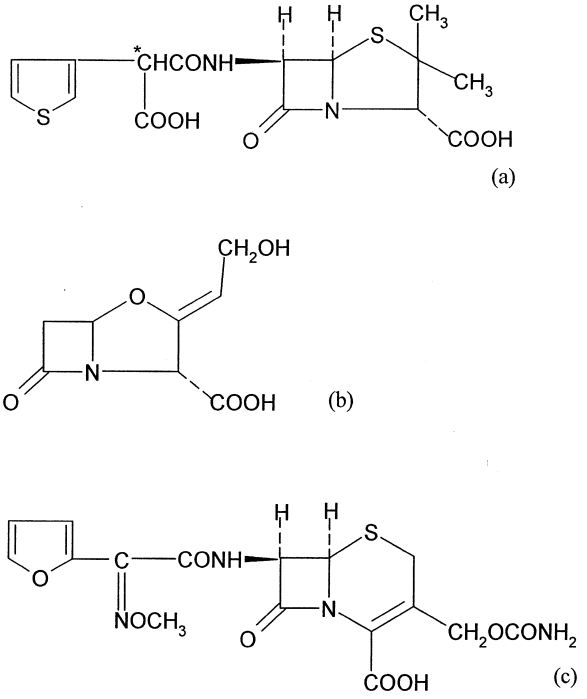

Fig. 1. Chemical structure of ticarcillin (a), clavulanate (b), and cefuroxime (c). Since ticarcillin is an isomer, the chiral center is the carbon labeled with * in ticarcillin. The internal standard is cefuroxime.

ticarcillin and clavulanate in biological fluids, including microbiological assay [4–7] and HPLC methods. These HPLC methods for ticarcillin assay included classical reversed-phase HPLC [8,9] and ion-pair HPLC [10–12]. Haginaka et al. [13] employed a precolumn reaction with 1,2,4-triazole and mercury(II) chloride to analyze ticarcillin in serum and urine. Due to isomerization of ticarcillin, two stereospecific HPLC methods have been developed [1,14]. Different sample handling procedures were employed in these methods, e.g. liquid–liquid extraction, solid-phase extraction, and ultrafiltration. The lowest of the limits of detection among these methods for ticarcillin alone is 0.5 μg/ml in serum and 1 μg/ml in urine at 205 nm.

Clavulanate has an ultraviolet absorption maximum at 201 nm in water, which causes difficulty in the assay of clavulanate under UV detection. Thanks to the bathochromic shifts by the ion pairing agent tetrabutylammonium bromide, clavulanate in urine samples was quantified by an ion pair reversed-phase HPLC method at 220 nm [15]. Precolumn reactions with imidazole [14,16], 1,2,4-triazole [17], benzaldehyde [18] and postcolumn techniques with alkaline degradation [19–22] were employed in HPLC methods to analyze clavulanate in biological fluids. Meanwhile, HPLC methods utilizing an UV detector were reported for the assay of clavulanate in pharmaceutical products of amoxicillin/clavulanate [23,24]. Moreover, capillary electrophoresis [25] and an HPLC method with electrochemical detection [26] were described to measure clavulanate and amoxicillin in pharmaceutical preparations.

So far there is no HPLC method available to simultaneously analyze ticarcillin and clavulanate in biological fluids. The purpose of the present study was to develop a simple, reproducible and selective HPLC method to simultaneously determine ticarcillin and clavulanate in biological fluids. This method was applied to analyze both compounds in rabbit serum and tissue cage fluids in a pharmacokinetic study.

2. Experimental

2.1. Chemicals

Ticarcillin and clavulanate were supplied by GlaxoSmithKline (West Sussex, UK), cefuroxime (internal standard) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ChromAR HPLC grade, Mallinckrodt, Muskegon, MI, USA) and dichloromethane (B&J Brand high purity solvent, Burdick and Jackson, Muskegon, MI, USA) were used without further purification. Deionized water was obtained from a Milli-Q Plus analytical deionization system (Bedford, MA, USA). Sodium dihydrophosphate monohydrate and phosphoric acid were obtained from Sigma and J.T. Baker (Phillipsburg, NJ, USA), respectively. The ion-pair chromatographic reagent, tetrabutylammonium hydrogen sulfate (TBAS), was purchased from Sigma.

2.2. Instrumentation

A HPLC system which consisted of a 626 gradient pump (Waters, Milford, MA, USA), a 600S controller (Waters), a 717 plus autosampler (Waters), and

LDC SM4000 programmable wavelength UV detector (LDC/Milton Roy, Riviera Beach, FL, USA) was used for this experiment. The separation was accomplished on a reversed-phase column, Waters Nova-Pak C18 column (150×3.9 mm, 4 μm) coupled to a μBondapak C18 Guard-Pak precolumn (Waters). The column was maintained at room temperature. The quantification was based on peak height using EZCHROM ELITE chromatography data system (Scientific software, San Ramon, CA, USA).

2.3. Chromatographic conditions

All chromatographic procedures were performed at ambient temperature. Two solutions were used in this gradient elution, solution A 80:20 (v/v) acetonitrile–phosphate buffer (pH 4.1, 0.023 *M*) with 4 m*M* TBAS, and solution B 3:200 (v/v) acetonitrile–phosphate buffer (pH 4.1, 0.023 *M*) with 4 m*M* TBAS. To de-gas the mobile phases, helium at 100 ml/min was bubbled through the solutions for the first 15 min, and then bubbled at 30 ml/min during the remainder of the running time. The chromatograms were performed with a parabolic gradient program, as follows: 0–10 min from 5% solution A to 35% solution A by gradient curve #8, 10–18 min return from 35% solution A to 5% solution A by linear change, 18–22 min remain at 5% solution A. The running time for one sample was 22 min. The mobile phase flow-rate was 1 ml/min. The UV detector was set at 218 nm with a sensitivity of 0.01 a.u.f.s. for the first 9 min, at 254 nm from 9 to 18 min, and at 218 nm from 18 to 22 min.

2.4. Standard solutions and quality controls

The stock solutions of ticarcillin (2.0 mg/ml) and clavulanate (0.4 mg/ml) were made in volumetric flasks in deionized water. The internal standard (I.S.) stock solutions, cefuroxime (2.0 mg/ml), were prepared in deionized water.

Rabbit serum and tissue cage fluid (TCF) (see Section 2.7) was collected from pooled blood and TCF obtained from drug-free rabbits. These biological fluids were centrifuged at 2000 *g* for 20 min. The obtained blank serum and TCF were stored at −20 °C before use. For the calibration curves of ticarcillin/clavulanate, a series of rabbit serum and TCF were spiked with both compounds to give a final concentration of 1.0, 5.0, 10.0, 30.0, 60.0 and 100.0 μg/ml of ticarcillin, and 0.2, 1.0, 2.0, 6.0, 12.0 and 20.0 μg/ml of clavulanate. Aliquots of 250 μl of these standard solutions were stored at −80 °C until analysis. Three quality control (QC) samples were prepared using the same procedure in rabbit blank serum and TCF, the concentrations of the controls are as follows: 2.0, 20.0 and 80.0 μg/ml of ticarcillin, and 0.5, 4.0 and 20.0 μg/ml of clavulanate.

2.5. Extraction procedure

The sample preparation was a two-step procedure [27]. A 0.2-ml aliquot of serum or TCF sample was pipetted into a clean tube, and spiked with 30 μl of internal standard (30 μg/ml cefuroxime in pH 7, 0.2 *M* phosphate buffer). Acetonitrile (0.8 ml) was added to precipitate the protein in the sample. The mixture was shaken vigorously for 5 min and then centrifuged at 2250 *g* for 5 min (IEC Centra-HN 2485, Needham Height, MA, USA). The supernatant was transferred to a clean tube, and 2 ml of dichloromethane was added. After 5-min vigorously mixing and 5-min 2250 *g* centrifugation, the top aqueous layer was aspirated for the HPLC assay. As we had run a preliminary study before, the concentration of the unknown samples above the quantification limits (100 μg/ml ticarcillin or 20 μg/ml clavulanate) were adequately prediluted with tested drug-free rabbit serum or TCF.

2.6. Assay validation

Calibration curves with six concentration points were constructed by plotting the peak ratio of ticarcillin or clavulanate to internal standard, cefuroxime, versus the theoretical concentrations. Weighted (1/concentration) least square linear regression analyses were applied to generate the calibration curves, which can be used to calculate the concentrations of the quality controls and the unknown samples. The Student's *t*-test was used to test the linearity of the standard curve, i.e. comparison of the slopes of the linear curves with zero, and the intercept with zero.

The lower limits of quantification (LLQ) of the assay were evaluated using six samples with 1 μg/

ml ticarcillin and 0.2 μg/ml clavulanate in rabbit serum and TCF. The precision and accuracy of this method were evaluated by assaying each low, middle and high concentration of the quality controls (2, 20, 80, μg/ml of ticarcillin, and 0.2, 4, 16 μg/ml of clavulanate). These three QC samples were also used to assess the recovery and stability. Recoveries were evaluated by comparing peak heights of ticarcillin, clavulanate and the internal standard from the extracted standards in rabbit serum and TCF with those for nonextracted standards in aqueous solutions. The stability of ticarcillin and clavulanate in rabbit serum and TCF at room temperature and at −80 °C was assessed. The stability of the samples after extraction was also evaluated while in the 10 °C autosampler.

2.7. Pharmacokinetics study

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Hartford Hospital (Hartford, CT, USA). Six female New Zealand white rabbits, weighing about 4 kg, were used in this study. All animals received water and food ad libitum. The animal vendor, Covance Research Product (Denver, PA, USA) implanted subcutaneously two golf wiffle balls on the rabbit dorsal cervical surface. The golf wiffle balls were used as the tissue cage, which are 2-mm thick, 4-cm diameter hollow plastic balls with an even distribution of 26 0.5-cm holes. During the 4-week surgical recovery period including a 1-week quarantine at the Hartford Hospital animal laboratory, the wiffle balls filled with fluid, this fluid is so-called tissue cage fluid (TCF) that served as a bacterial growth medium.

To develop a soft tissue infection, rabbits with implanted wiffle balls for 4 weeks were infected by percutaneously injecting 2 ml suspension of *P. aeruginosa* PSA246 (10^6 cfu/ml) into each wiffle ball. After 24 h of organism incubation in the wiffle balls, the rabbits were subcutaneously injected with a single dose of 400 mg/kg of ticarcillin and 13.3 mg/kg of clavulanate (Timentin®). Blood was sampled by using marginal ear vein bleeding technique, the sampling time points were as follows: 0 (prior to the injection), 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h after dosing. The blood samples (1 ml at each time point) were centrifuged, separated and frozen at −80 °C until analysis. TCF (0.4 ml at each time point) was aspirated percutaneously at 0 (prior to the injection), 1, 2, 4, 6, 8 and 12 h after dosing. TCF samples were directly stored at −80 °C without processing.

3. Results

3.1. Retention times and specificity

Figs. 2 and 3 represent the typical chromatograms of blank rabbit serum and TCF, ticarcillin, clavulanate and internal standard in these matrixes. The chromatogram shows that no endogenous peaks interfered with ticarcillin, clavulanate and cefuroxime in rabbit serum and TCF. After ion-pairing with TBAS, the retention times of clavulanate, cefuroxime and ticarcillin were 5.1, 14.7 and 15.6 min, respectively. The running time for each sample was 22 min.

3.2. Linearity

The weighted linear regression of peak height ratio of the compound of interest in rabbit serum and TCF over the internal standard versus concentration was conducted. The linearity of these four standard curves was tested statistically. For each calibration curve, the slope was statistically different from zero ($P<0.05$), and the intercept was not statistically different from zero ($P>0.05$). The correlation coefficient (r) for each calibration curve was >0.999. The summary data of correlation coefficient, slope and intercept of the standard curves of ticarcillin and clavulanate in rabbit serum and TCF are shown in Table 1.

3.3. Lower limit of quantification

The precision of LLQ samples was determined by the relative standard deviation (RSD) of mean value; the accuracy of LLQ samples was evaluated by the relative error from the theoretical concentrations. The precision and accuracy of LLQ of ticarcillin were 3.24 and 5.13% in serum, and 5.98 and 6.90% in TCF, respectively. The precision and accuracy of

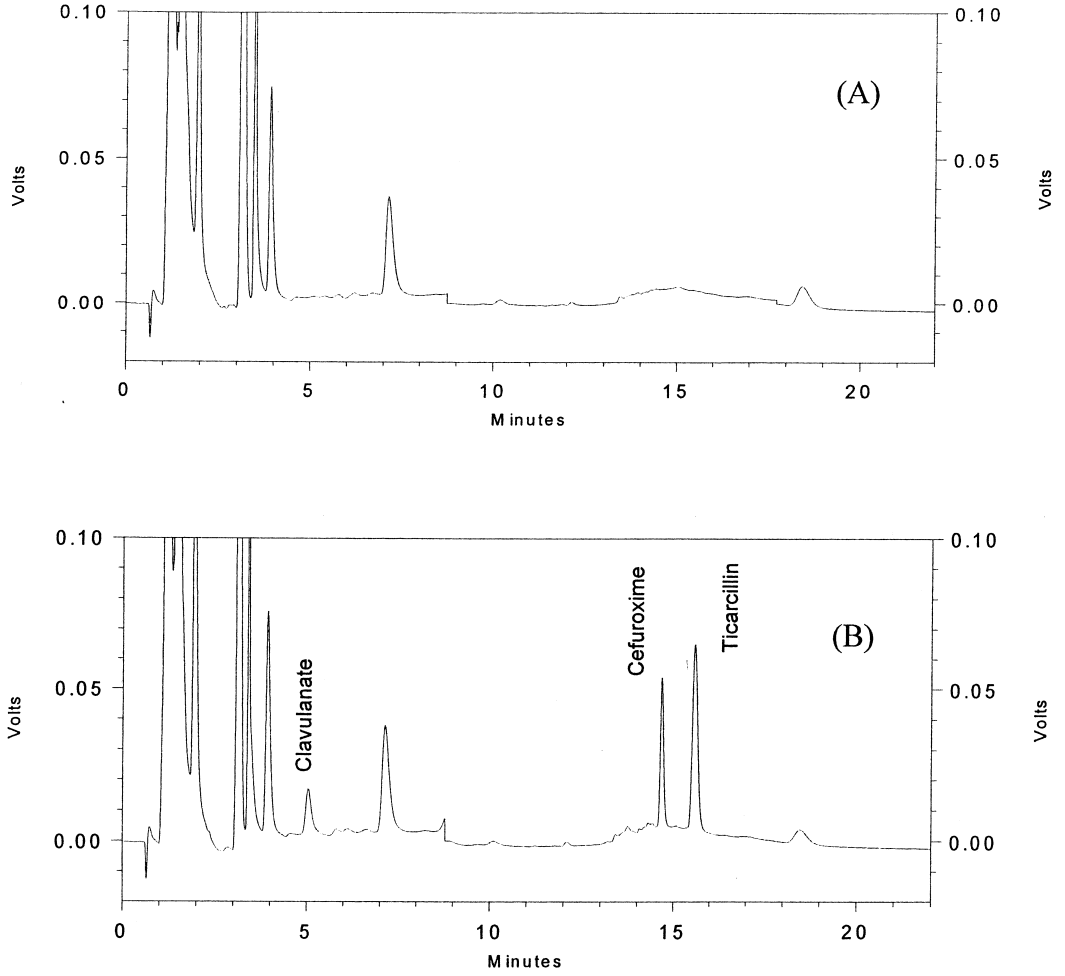

Fig. 2. Chromatograms for (A) blank serum and (B) the serum obtained from a rabbit at 6 h after subcutaneous injection of 400 mg/kg ticarcillin and 13.3 mg/kg clavulanate.

LLQ of clavulanate were 5.65 and 4.57% in serum, and 3.65 and 7.49% in TCF, respectively.

3.4. Precision and accuracy

Between- and within-batch variations of these assays for low, medium and high concentration of quality controls in rabbit serum and TCF were determined using frozen controls. The precision of the assay was determined by the RSD of the mean value from the theoretical concentration; the accuracy of the assay was evaluated by the relative error of the backcalculated concentration from the theoretical concentration. The between-batch precision of three QC samples of ticarcillin in rabbit serum and TCF was from 1.54 to 6.03%, the within-batch precision of ticarcillin was from 0.75 to 2.97%. The between-batch accuracy of three QC samples of ticarcillin in rabbit serum and TCF was from 0.25 to 2.75%, the within-batch accuracy of ticarcillin was from 0.43 to 4.54% (Table 2). The between-batch precision of three QC samples of clavulanate in rabbit serum and TCF was from 1.59 to 4.74%, the within-batch precision of clavulanate was from 1.25 to 3.95%. The between-batch accuracy of three QC samples of clavulanate in rabbit serum and TCF was from 0.42

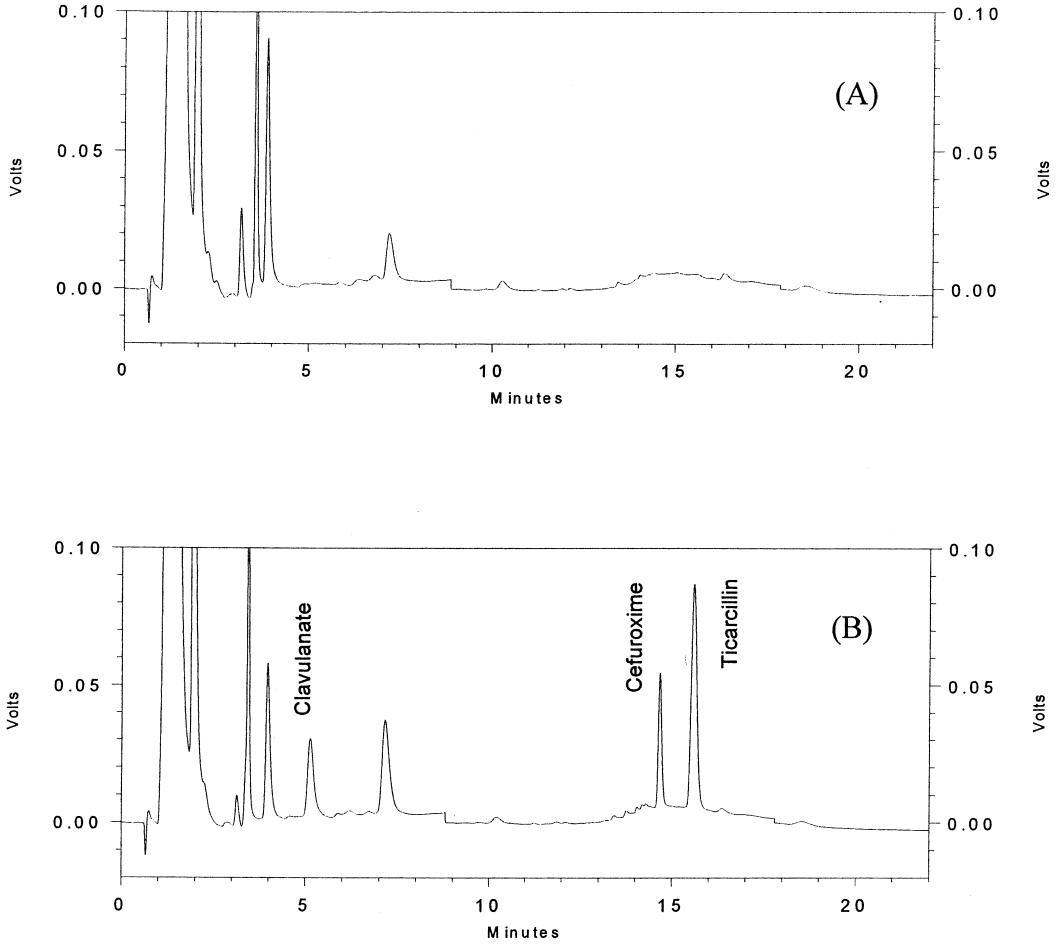

Fig. 3. Chromatograms for (A) blank TCF (tissue cage fluid) and (B) the TCF obtained from a rabbit at 1 h after subcutaneous injection of 400 mg/kg ticarcillin and 13.3 mg/kg clavulanate.

to 5.50%, the within-batch accuracy of clavulanate was from 3.09 to 7.25% (Table 3). All of RSDs and relative errors of ticarcillin and clavulanate in rabbit serum and TCF were <10%.

3.5. Recovery

The percentage recovery for the compound of interest from biological matrices versus water was

Table 1
Assay linearity of ticarcillin and clavulanate in rabbit serum and TCF

Sample	Corr. coefficient (mean±SD)	Slope (mean±SD)	Intercept (mean±SD)
CLA in serum[a]	0.9994±0.0005	0.0947±0.0041	0.0041±0.0046
CLA in TCF[b]	0.9998±0.00003	0.1199±0.0045	0.0068±0.0051
TIC in serum[a]	0.9996±0.0002	0.0208±0.0011	0.0107±0.0036
TIC in TCF[b]	0.9997±0.0002	0.0224±0.0010	0.0108±0.0053

[a] $n=14$.
[b] $n=12$.

Table 2
Precision and accuracy of the assay of ticarcillin in rabbit serum and TCF

Theoretical conc. (μg/ml)	*n*	Calculated conc. (μg/ml)	SD	RSD (%)	Relative error (%)
Ticarcillin in serum					
Intra-batch					
2	10	2.09	0.06	2.97	4.49
20	10	20.24	0.42	2.07	1.22
80	10	80.34	0.77	0.95	0.43
Inter-batch					
2	13	2.01	0.09	4.32	0.25
20	13	20.28	0.48	2.35	0.42
80	13	80.32	1.24	1.54	0.39
Ticarcillin in tissue cage fluid					
Intra-batch					
2	10	2.09	0.05	2.17	4.54
20	10	20.10	0.36	1.77	0.48
80	10	78.46	0.59	0.75	1.93
Inter-batch					
2	11	2.01	0.12	6.03	0.40
20	11	20.55	0.74	3.60	2.75
80	11	80.16	1.65	2.06	0.20

measured by comparing the peak heights. The mean recoveries of ticarcillin in serum and TCF, clavulanate in serum and TCF, were calculated as 76.81±3.16, 79.93±5.71, 75.77±4.87 and 83.44±4.51, respectively (n=9). There was no difference in the recoveries at three various concentrations. The mean recovery of the I.S. in serum and TCF were 111.32±0.46 and 108.30±1.03, respec-

Table 3
Precision and accuracy of the assay of clavulanate in rabbit serum and TCF

Theoretical conc. (μg/ml)	*n*	Calculated conc. (μg/ml)	SD	RSD (%)	Relative error (%)
Clavulanate in serum					
Intra-batch					
0.4	10	0.43	0.01	1.31	7.25
4	10	3.76	0.12	3.13	6.01
16	10	16.77	0.66	3.95	4.84
Inter-batch					
0.4	13	0.41	0.02	4.63	3.00
4	13	3.78	0.15	3.88	5.50
16	13	15.73	0.75	4.74	1.69
Clavulanate in tissue cage fluid					
Intra-batch					
0.4	10	0.39	0.01	3.29	3.09
4	10	3.77	0.05	1.29	5.74
16	10	15.46	0.19	1.25	3.39
Inter-batch					
0.4	11	0.397	0.02	3.82	0.75
4	11	3.92	0.06	1.59	2.10
16	11	16.07	0.38	2.35	0.42

tively (n=9). No effect of the co-extracted biological material was detected.

3.6. Stability

Ticarcillin and clavulanate were stable, almost no change in rabbit serum and TCF at −80 °C for at least 2 months. After bench top storage at room temperature for 6 h, ticarcillin and clavulanate in rabbit serum and TCF were stable with <5% degradation. Run-time stability study showed that after extraction, ticarcillin and clavulanate in rabbit serum and TCF were stable, <5% degradation at 10 °C in the autosampler for up to 26 h. There was a <10% loss for ticarcillin and clavulanate in rabbit serum and TCF after three freeze–thaw cycles.

3.7. Animal study

This assay was used for the determination of ticarcillin and clavulanate in rabbit serum and TCF for a pharmacokinetics study. Fig. 4 represents a plot of concentrations of ticarcillin and clavulanate versus time in rabbit serum. Fig. 5 represents a plot of concentrations of ticarcillin and clavulanate versus time in rabbit TCF. Obviously, the concentration–time profiles of both compounds in rabbit serum were different from those in TCF.

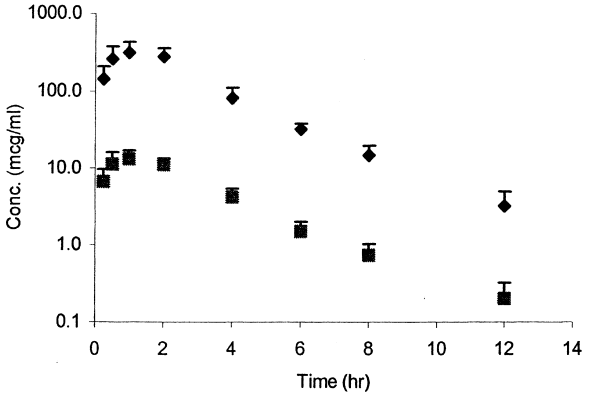

Fig. 4. Concentrations of ticarcillin (♦) and clavulanate (■) in rabbit serum following subcutaneous injection of Timentin® (400 mg/kg ticarcillin and 13.3 mg/kg clavulanate) to the infected New Zealand white rabbits. Each point represents the mean value (n=6); the upper side bar represents the standard deviation.

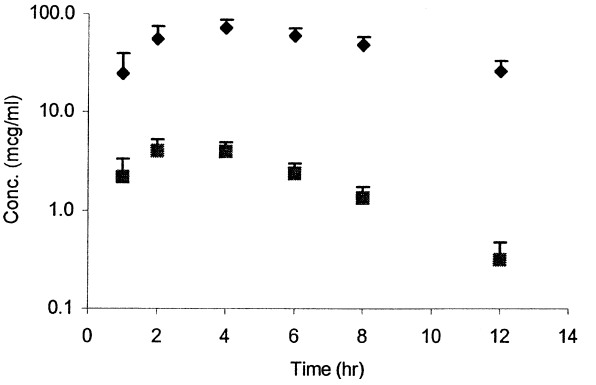

Fig. 5. Concentrations of ticarcillin (♦) and clavulanate (■) in rabbit TCF (tissue cage fluid) following subcutaneous injection of Timentin® (400 mg/kg ticarcillin and 13.3 mg/kg clavulanate) to the infected New Zealand white rabbits. Each point represents the mean value (n=6); the upper side bar represents the standard deviation.

4. Discussion and conclusions

There are few ultraviolet absorbing chromophores in clavulanate, which has no ultraviolet absorption peak over 210 nm. Due to low wavelength absorption, it is difficult to develop a HPLC method for the UV detection of clavulanate in biological fluids. The interference from endogenous peaks is the major issue for developing an assay to measure clavulanate in the biomatrix, the sensitivity and selectivity of the assay become low, and the detection limit of clavulanate in human urine is as high as 5 μg/ml. In order to improve the sensitivity of the HPLC assay, time-consuming precolumn and postcolumn derivatization procedures were applied with an UV or fluorimetric detector. In this method without any derivatization, the use of the ion-pairing reagent tetrabutylammonium hydrogen sulfate prolongs the retention time of clavulanate from 2 to 5 min with a sharp peak, and fortunately there is no competing endogenous peak at 5 min. The limit of quantification of clavulanate in this assay can reach to 0.2 μg/ml in rabbit serum and TCF.

Chemically, ticarcillin is a mixture of two epimers (R and S), the chiral center is the α-carbon in the function group of α-carboxy-3-thienylacetamido, which is labeled in Fig. 1a. Spectroscopically, like clavulanate, ticarcillin lacks strong absorption chromophores in its structure. Therefore the inherent

problems of a ticarcillin assay are low sensitivity and peak splitting. Follette et al. [10] solved the low sensitivity problem by setting the wavelength as low as 205 nm to push the limit of quantification to 0.5 μg/ml, and added the ion-pairing reagent tetramethylammonium chloride to the mobile phase to prevent the peak splitting of ticarcillin. However, since there is no stereoselectivity in the pharmacokinetics of ticarcillin [1], it is not necessary to develop a stereoselective HPLC method to measure ticarcillin. The use of the ion-pairing reagent tetrabutylammonium hydrogen sulfate in the present assay also can reduce the column selectivity to ticarcillin, and then ticarcillin presents a single sharp peak in the chromatogram. Thanks to the very sharp peak, the limit of quantification of ticarcillin can reach 1 μg/ml even at a wavelength of 254 nm.

No HPLC method has been reported to simultaneously measure ticarcillin and clavulanate in biological fluids, because ticarcillin and clavulanate have different chemical structures and different UV characteristics, plus the difficulty of assaying clavulanate. As the UV wavelength was fixed at 218 nm, endogenous peaks interfere with ticarcillin and the I.S. even using totally different mobile phases and different gradient elution programs. Derivatization of clavulanate may partly or completely degrade ticarcillin, the simultaneous quantification of ticarcillin become unreliable. On the other hand, it is not sensitive enough to measure clavulanate levels while the UV detector was set at a high wavelength, that is the approach many published assays used to determine ticarcillin alone. Changing the wavelength from 218 to 254 nm at 9 min not only provided the optimal UV absorption intensity for both compounds, but also eliminated the endogenous interference with ticarcillin and the I.S. from the matrix. No matter at what wavelength the compound of interest was monitored, the I.S. (cefuroxime) had a stable UV absorption intensity. This guaranteed that the simultaneous determination of ticarcillin and clavulanate could be achieved using the I.S. method. Such switching wavelength technique in gradient elution HPLC had been demonstrated successfully in the simultaneous analysis of another combination of β-lactam/β-lactamase inhibitor, piperacillin and tazobactam in rabbit serum and TCF [28].

In the present study, a new analysis was developed to simultaneously determine ticarcillin and clavulanate in biological fluids. This assay is simple, accurate and reproducible. This method may be applied to analyze ticarcillin and clavulanate in pharmaceutical products. For further applications of this assay, this method with the wavelength switch technique may be applied to analyze amoxycillin and clavulanate in biological fluids after modification of the mobile phase.

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