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Determination and Pharmacokinetics Study of β-Lactamase in Rat Plasma by Fluorimetric HPLC

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Abstract

For the determination of in vivo β-lactamase activity, a high-performance liquid chromatographic (HPLC) method was established, and the pharmacokinetics of β-lactamase after intravenous administration to the rats was analyzed using this standardized HPLC method. The plasma samples containing β-lactamase were reacted with ampicillin (substrate) and further processed to make them fluorescent. The fluorescent compound of interest was separated using HPLC at room temperature using the excitation and the emission wavelengths of 410 nm and 475 nm, respectively. For the pharmacokinetic studies, 252 mU of β-lactamase solution was administered to the rats through the tail vein injection (n = 6). The blood samples were withdrawn from the tail vein at different time points and analyzed by HPLC for β-lactamase activity. For the HPLC method of β-lactamase in plasma samples, the peak area showed a good correlation within the concentration ranges of 0.126–12.6 mU/mL (10–1000 ng/mL). The coefficients of variations were within 0.56–6.24, and the percentage recovery were within 102–107. After the intravenous injection, plasma concentration at the time zero (Cp_0) was 11.47 ± 0.48 mU/mL, and no β-lactamase was detected 24 h after the injection. The volume of distribution (V_d) was 22 mL. An elimination half-life ($t_{1/2}$) of 4.12 ± 0.5 h and AUC of 79.4 ± 12.9 mU.hr/mL were also calculated. The HPLC fluorimetric method was a very sensitive and reproducible method for the detection of β-lactamase in plasma. The disposition of β-lactamase after intravenous administration followed one-compartment and first-order kinetics.

Introduction

β-Lactamases are the enzymes that catalyze the hydrolysis of β-lactam ring (Figure 1) and are responsible for the bacterial resistance to penicillin, cephalosporin, and many other antibiotics. They are produced by some of the gram-positive as well as gram-negative bacterium. β-Lactamases are structurally related to the enzymes involved in cell wall synthesis, the so-called penicillin-binding proteins (1). The size of these enzymes is 29 kDa; structurally it is organized in two domains (2). More than 190 β-lactamases are known so far (3). TEM-1 is the most widespread β-lactamase and is accountable for most of the ampicillin resistance in different bacterium (4). This β-lactamase was named after the initials of the patients from which this β-lactamase enzyme was isolated (5).

In most of the frequently used antibiotics, like penicillin, cephalosporin, and their derivatives, a β-lactam group is present, making β-lactamases clinically very important. The expression of β-lactamases by bacteria helps them to develop resistance to the antibiotics as these enzymes activate the hydrolysis of β-lactam containing antibiotics. With the rapid appearance of resistance to existing antibiotics, there is a huge demand for new antibiotics. Thus it is very important to understand the extent and the effect of β-lactamase resistance in order to extend the recent broad-spectrum β-lactam antibiotics. Also, the most important factor affecting the pharmacodynamics of β-lactamase inhibitor–β-lactam combinations is the maintenance of a critical concentration of inhibitor crucial to sufficiently suppress β-lactamase activity (6). One of the critical factors that determine this concentration would be directly dependent upon the amount and type of β-lactamase produced. Consequently, understanding the effect and extent of β-lactamase resistance is important for the development of broad-spectrum β-lactam antibiotics and/or the inhibitor-drug combinations. Recently, the significance of β-lactamase to prevent infection when administered orally was explored by Stiefel et al. (7). Based on their study, it was concluded that when β-lactamase is administered in conjugation with the parenteral administration of β-lactam antibiotics, β-lactamase tend to preserve the natural intestinal flora. Thus, the precise determination of β-lactamase in the biological samples can be very decisive in both non-clinical and clinical phases of drug discovery.

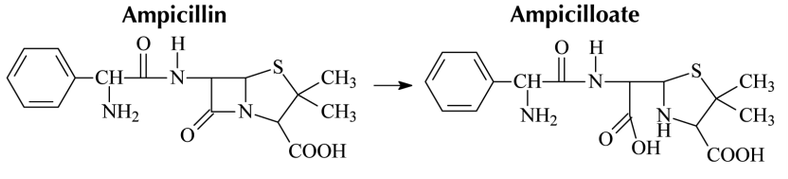

Figure 1. Hydrolysis of the β-lactam ring by β-lactamase enzyme.

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The methods that have commonly been used for the assay of β-lactamase include iodometric (8), spectrophotometric (9), nitrocefin test (10), acidimetric test (11), fluorimetry (12), microbiological test (13), and high-performance liquid chromatography (HPLC) (14–17). All these assay methods, except HPLC, detect the presence and/or the secretion of high concentrations of β-lactamase and also require high substrate concentrations. Thus, these methods are not suitable to detect the clinical amounts of β-lactamase in the body. Ross et al. (18) have compared iodometric, spectrophotometric, acidimetric test, and microbiological test for the assay of β-lactamase. They have concluded that UV–spectrophotometric is the method of choice among these methods. The significance of the HPLC methods for the assays of enzyme activities has been reviewed by Lambeth and Muhonene (19). To date, all the available HPLC methods use the in vitro samples and are not sensitive enough to analyze the clinically significant concentrations of β-lactamase in blood. Most of these methods measure the catalytic conversion of reactants to product and correlate that with the amount of β-lactamase.

In the present study, we have developed an HPLC method, employing a fluorescent detector for the detection of TEM-1 β-lactamase in vivo to as low as 0.126 mU/mL (10 ng/mL). According to Baker (20), β-lactamase hydrolysis converts ampicillin to ampicilloate (Figure 1), which under specific conditions gives a fluorescent product. Baker (12) further reported a fluorimetric method for determination of β-lactamase activity in a bacterial suspension. After ampicillin is converted to penicilloate, the sample is treated with sodium hydroxide, neutralized, diluted with 0.5 M acetate buffer at pH of 4, and heated for 30 min at 100°C in the presence of ascorbic acid and EDTA. This procedure was successfully used in our lab to analyze the β-lactamase in the in vitro samples up to the minimum quantitation limit of 1 mg/mL (21). The same procedure was tested on the plasma samples containing β-lactamase, although without any success. Even at the similar or higher β-lactamase concentrations of 1 mg/mL, a potential interference was observed from the spiked plasma samples using this reaction and analyzing the samples by this fluorimetric technique.

To determine the β-lactamase activity in plasma, this procedure should be customized to increase the selectivity of the assay and decrease any possible interference. HPLC method as a technique to assay enzymatic reactions has various advantages (19). For the prospective of the present study, HPLC method can increase the sensitivity of assay, which will enable us to establish a robust analytical method for the detection of clinically significant β-lactamase quantities in the body. As defined by Baker (22), the hydrolysis of antibiotics by β-lactamase results in product, which can be read under fluorimeter. By monitoring the amount of this product, the enzyme concentration can be extrapolated. In our in vitro method, the enzyme concentration was successfully monitored, and a standard plot was generated based on this reaction. In order to further enhance the sensitivity of this enzyme assay, separating the fluorescent product from the complex mixture can be accomplished by injecting the sample into the HPLC system. The significance of separating substrate and products and quantifying the extent of reaction by HPLC has been covered comprehensively by Lambeth and Muhonen (19). These separated compounds were detected and quantified on the fluorescence detector connected with the HPLC system.

Some bacteria have the capability to produce β-lactamase in biological fluids, but β-lactamase is never present in those individuals who have not been recently exposed to the antibiotics (23). Thus the pharmacokinetics of β-lactamase may vary in the presence or absence of a particular bacterium. It seems to be of great interest to know the pharmacokinetics of β-lactamase in vivo in order to understand how the antibiotics are being metabolized by these enzymes. This can further help in comprehending the extent of bacterial resistance and some suitable measures that can be taken to avoid or minimize these effects. The literature search shows that there is a lack of information on the pharmacokinetics of β-lactamase. Therefore, in the present study, we have investigated the pharmacokinetic profile of β-lactamase after intravenous administration through the tail vein to the female Sprague-Dawley rats using the presently optimized fluorimetric HPLC method. In order to reduce the variability, only those animals that have never been exposed to any antibiotic were used for the present study.

Experimental

Chemical and reagents

All chemicals were analytical grade. Ampicillin, TEM-1 β-lactamase (from Bacillus cereus EC 3.5.2.6), ascorbic acid, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Fisher Chemicals (Fair Lawn, NJ).

Female Sprague-Dawley rats weighing 325 ± 25 g, purchased from Taconic Farms (Germantown, NY) were used in these experiments. The rats were acclimated to their surrounding for at least 1 week and were housed in the same room. The micro-isolator cages were used with two animals in each cage. They were housed on a 12 h light/dark cycle and a relative humidity of 40–60%.

Blank, drug-free plasma was also collected from these animals. The blood was collected in an Eppendorf tube to which a pinch of EDTA (anticoagulant) had been added. The plasma was immediately separated from erythrocytes by centrifugation at 9000 × *g* for 3.5 min, and plasma was collected in a separate Eppendorf tube.

Instrumentation and chromatographic conditions

Fluorescence spectrophotometer

Luminescence Spectrometer LS 50B (PerkinElmer, Wellesley, MA) equipped with FL WinLab software was used for the fluorescence spectrophotometry.

HPLC system

All the chromatographic studies were performed on a Waters HPLC system (Milford, MA) and consisted of a Waters 600E system controller, a Waters 717 autosampler, and a Waters 470 scanning fluorescence detector. The separations were done on a Bondapak C_{18} cartridge column (300 × 3.9 mm i.d.) with a column particle diameter of 37–55 µm. The column effluents were monitored at an excitation and an emission wavelength of 410 nm and 475 nm, respectively, for a run-time of 11 min. The

mobile phase was 80% of 0.1 M potassium dihydrogen phosphate buffer (pH = 5) and 20% acetonitrile. The mobile phase was filtered and degassed before use. The flow rate was 1.5 mL/min, and the injection volume was 10 µL.

Preparation of stock and standard solutions

A stock solution (0.1 mg/mL) of β-lactamase was prepared in the phosphate buffer saline (PBS). This stock was serially diluted in the rat plasma to give working standard solutions of 0.126, 0.378, 0.630, 1.26, 3.78, 6.30, and 12.6 mU/mL (equivalent to 10, 30, 50, 100, 300, 500, and 1000 ng/mL, respectively). The stock solution of ampicillin (substrate) was freshly prepared in PBS (6.25 mM). In order to investigate the impact of biomatrix (proteins and endogenous substances) on the product formation, a blank plasma sample was also used in this study. All the plasma solutions were protected from light and were stored at –20°C. Each sample was made in triplicate, and the samples were prepared and processed on three different days.

The samples were processed as described by Baker (12) with some modifications. Using their reaction mechanism, the volume of different reagents that were used in the following reaction scheme was adjusted in order to account for the sensitivity of the procedure. To a 0.2 mL of β-lactamase sample, 0.2 mL of ampicillin solution (6.25 mM) was added. The reaction mixture was then incubated at 37°C for 10 min. To the reaction mixture, 1 mL of pre-warmed 0.5 M acetate buffer (pH = 4, containing 100 mM EDTA and 1.0 mg/mL ascorbic acid) was immediately added. This solution was heated at 100°C for exactly 30 min. The samples were allowed to cool to room temperature for 1 h and were centrifuged at 9000 × *g* for 2 min. The samples were stored at 2–6°C until further analysis.

All the blood samples were collected in an Eppendorf tube, and the plasma was recovered from these blood samples by the procedure outlined earlier. The β-lactamase in these plasma samples was analyzed by the HPLC method.

Selection of excitation and emission wavelength

The samples with varying concentrations of substrate and/or enzyme were prepared and then processed as discussed earlier. A full-range fluorescence scan of each of these samples was done by the luminescence spectrometer in order to recognize the peak excitation and emission wavelengths. The scans of wavelength (200–800 nm for excitation and 200–900 nm for emission) were done using slit widths of 10 nm and 5 nm for excitation and emission, respectively. The scan speed of 480 nm/min was used. The photomultiplier voltages were set nominally by the instrument to a voltage dependent on the excitation slit position and the position of the excitation monochromator. Fluorescence intensity of the different samples was also measured at the selected wavelengths under the fluorescence spectrophotometer.

Method validation

Three quality control samples (0.315, 3.15, and 9.45 mU/mL) were processed, and each of these samples was analyzed three times (inter-day variation) on three different days (intra-days variation). These were labeled as low quality control (LQC) (0.315 mU/mL), medium quality control (MQC) (3.15 mU/mL), and high quality control (HQC) (9.45 mU/mL). The accuracy was calculated at each concentration as the ratio of the measured concentration to the nominal concentration multiplied by 100%.

The limit of quantitation (LOQ) of the method was defined as the lowest concentration that could be quantitatively determined with an acceptable precision and accuracy. The acceptable limits were defined as accuracy of 80–120% and precision of ≤ 20%. Stability of the processed samples was assessed by means of spiked β-lactamase samples stored under different temperatures.

Intravenous administration

The research protocol was approved by Institutional Animal Care and Use Committee (IACUC) of the St. John's University and adhered to the NIH guide for the use and the care of laboratory animals. The rat in vivo pharmacokinetic study was performed to confirm the applicability of newly developed and validated bioanalytical method. This study was performed on female Sprague-Dawley rats (n = 6, weight range 300–350 g). To each of the rats, a bolus dose of 0.2 mL of PBS containing 252 mU of β-lactamase was injected through the tail vein. Approximately 0.4 mL of blood was withdrawn from the tail vein before and at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h after the intravenous injection. All blood samples were collected in an Eppendorf tube, and the plasma was recovered from these blood samples as per the procedure outlined earlier. After each blood withdrawal, the volume was replaced by equal volume of intravenous administration of isotonic Ringer solution.

Pharmacokinetic parameters

All the pharmacokinetic parameters were analyzed from the concentration versus time profile. The plasma concentration-time data was plotted, and the area under this curve (*AUC*) from the beginning to the last measured plasma concentration (C_{last}), $AUC_{0\text{-}last}$, was determined by the linear trapezoidal method, and C_{last}/K_e was calculated as the $AUC_{last\text{-}\infty}$ (from last concentration time point to infinity). The total *AUC* was the sum of $AUC_{0\text{-}last}$ and $AUC_{last\text{-}\infty}$. The elimination rate constant (K_e), elimination half-life ($t_{1/2}$), plasma concentration at the time zero (Cp_0), apparent volume of distribution (V_d), and clearance from the blood (*Cl*) were calculated by the usual procedures (24). Statistical analysis was performed using Student's t-test with $\alpha = 0.05$ as the minimal level of significance.

Results and Discussion

Selection of wavelength and peak identification

In order to screen the wavelengths for the HPLC assay, three different types of samples were first read using the luminescence spectrometer: ampicillin and β-lactamase (substrate and enzyme); ampicillin (substrate) alone; and β-lactamase (enzyme) alone. Each of these samples was processed as described earlier. The sample containing β-lactamase and ampicillin showed a maximum excitation and emission wavelengths of 410 and 475 nm, respectively (Figure 2A–2B) shows that the peak excitation and emission wavelengths of the sample containing ampicillin alone were 365 nm and 427 nm, respectively. The sample having only β-lactamase showed no fluorescence. Thus, it can be con-

cluded from these scans that the processing procedure could make ampicillin fluorescent but not β-lactamase.

The fluorescence intensity of the samples was also recorded at the excitation and emission wavelength values of 365 nm and 427 nm, respectively. The fluorescence intensity of the samples containing 6.25 mM of ampicillin at these wavelengths was 21. This intensity value increase from 8 to 278 with the increasing concentration of enzyme (β-lactamase concentration range of 0.0126–126 mU/mL) to the fixed amount of substrate (6.25 mM of ampicillin). At the excitation and the emission wavelengths of 410 nm and 475 nm, respectively, the sample containing just the substrate (6.25 mM ampicillin) showed a fluorescent intensity of 12; the addition of 0.0126–126 mU/mL β-lactamase increased the intensity to 30–553. The sample in which β-lactamase and ampicillin are both present shows higher intensity as compared to the sample with substrate alone, and this fluorescence intensity increases with the increase in the amount of enzyme or the amount of substrate (sample with substrate alone). The interference due to ampicillin was minimal at the excitation and emission wavelength values of 410 nm and 475 nm, respectively. Baker (12) had taken into account the effect of internal florescence due to the ampicillin when calculating the contribution of florescence from the product (ampicilloate). For our studies, to minimize the interference the excitation and emission values 410 nm and 475 nm, respectively, were selected, and these wavelengths were used for HPLC analysis.

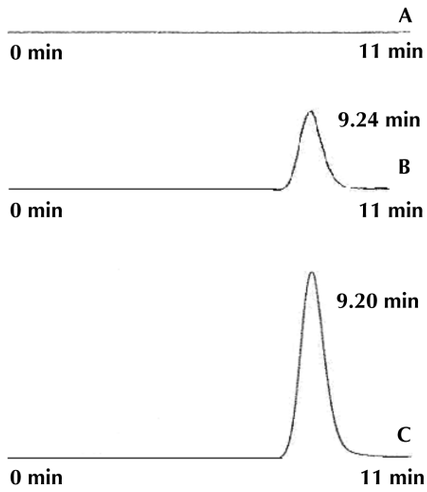

Figure 3. Representative HPLC chromatograms of the samples containing (A) β-lactamase alone; (B) ampicillin alone; and (C) ampicillin and β-lactamase. The samples were processed as described in the text and analyzed by HPLC.

The samples containing ampicillin and β-lactamase; ampicillin alone; and β-lactamase alone were processed and analyzed by the HPLC. Figure 3 shows the representative chromatograms of these three different types of samples. The samples having ampicillin alone and ampicillin with β-lactamase showed a peak at the retention time of 9.2 min when monitored at the excitation and the emission wavelengths of 410 and 475 nm, respectively. As discussed earlier, hydrolysis of the ampicillin leads to the formation of ampicilloate (Figure 1). The samples containing ampicillin alone had the same peak because ampicillin was hydrolyzed slightly, even though no β-lactamase was present. To further validate this hydrolysis, the samples containing ampicillin alone were first treated with 0.1 N NaOH to hydrolyze the ampicillin and then processed and analyzed by the HPLC. The obtained chromatogram from these samples was similar to the samples without NaOH hydrolysis, but the 9.2 min peak area increased significantly (from 73,800 to 790,666 by the NaOH hydrolysis). Thus, it can be concluded that it is the hydrolytic product of ampicillin that becomes fluorescent under specific conditions.

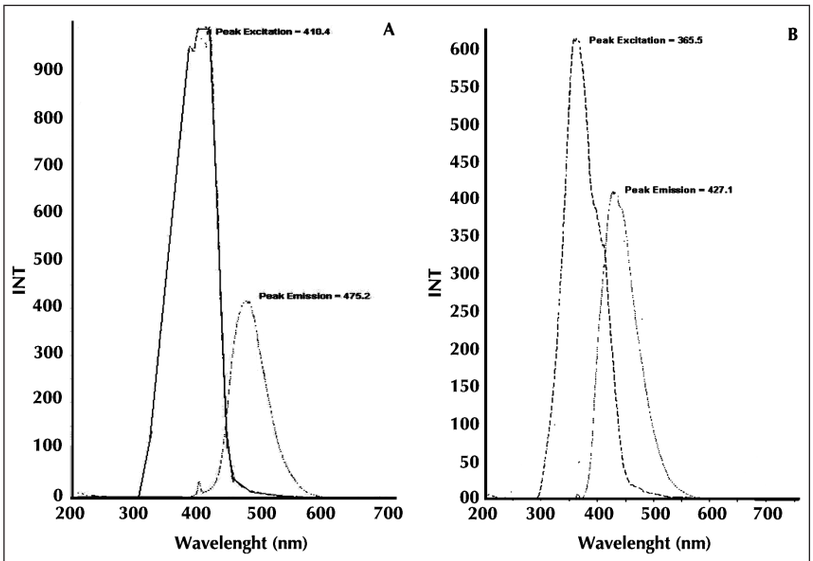

Figure 2. Fluorescence scans of the samples under spectrofluorometer: (A) β-lactamase and ampicillin and (B) ampicillin only. Plasma samples (200 µL) were allowed to undergo enzymatic reaction with 200 µL of ampicillin solution (6.25 mM), and the samples were processed as described in the text.

Standard curve and method validation

In order to optimize the concentration of ampicillin for our further studies, a fixed amount of β-lactamase was allowed to react with varying concentrations of ampicillin, and the samples were processed as described previously and analyzed by HPLC. By using this fixed amount of β-lactamase (1 µ/mL), peak area increased with the increasing concentration of ampicillin (data not shown). The increase in the peak area was larger when β-lactamase is present with ampicillin as compared to the area when ampicillin is present alone. The ampicillin does have some internal fluorescence, and it should be taken into account while validating this method. The ampicillin (substrate) concentration of 6.25 mM was selected for the reaction mixture in our further studies. This concentration value of ampicillin also made sure that there was sufficient substrate for the β-lactamase to react and at the same time minimized the interference from the internal fluorescence value of ampicillin.

The blank plasma was spiked with different concentrations of β-lactamase. This sample was then processed as outlined earlier and subjected to the HPLC assay. Based on the 9.2-min peak area obtained, a net peak area versus concentration plot was prepared (Figure 4). The peak area from the sample containing just 6.25 mM ampicillin (no β-lactamase) was subtracted from each of the peak area to get the net peak area. The curve was divided into two regions: 0.126–1.26 mU/mL and 1.26–12.6 mU/mL. The lowest β-lactamase concentration that can be quantitated with this assay method was 0.126 mU/mL, which is equivalent to 10 ng/mL. Linearity was exhibited in both these concentration ranges by this method. The average coefficient of determination of 0.99 was observed for all the three curves in both the concentration ranges. The slopes of these curves illustrated an excellent agreement with coefficient of variability of less than 0.45% for both the concentration ranges.

The intra- and inter-day relative standard deviations (RSD) were calculated to be 3.18% and 4.59%, respectively. Table I shows the accuracy and precision of the HPLC method at the three concentrations (0.315, 3.15, and 9.45 mU/mL). The coefficient of variations (CVs) of 6.237, 2.762, and 0.564% were obtained for these concentration samples. For the each concentration studied, a relative error (RE) of less than 10% was obtained. An acceptable precision and accuracy was acquired by this method for all the standards and quality controls based on the recommended criteria (25).

No peaks were seen in the chromatograms of the plasma samples of the undosed rats, which show a good selectivity of this method and rules out any potential interference of the biomatrix on the product formation.

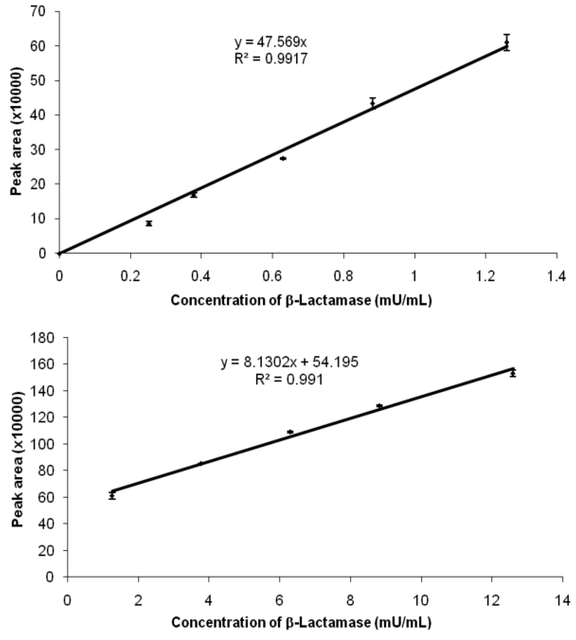

Figure 4. Standard curves for the HPLC assay of β-lactamase in plasma. The samples were processed as described in the text. For each concentration point, three samples were prepared, and each set of three samples was analyzed on three different days.

Stability of the samples

The stability of the samples during the analysis and during their storage at 2–6°C was also analyzed. No change in peak area was observed by injecting the same samples at the beginning and end of the run. If the samples were allowed to sit at room temperature, there was at least 10% increase in the peak area after 24 h, as determined by the HPLC assay. However, there was no change in the peak area if the samples were stored at 2–6°C for even one week. Thus, the samples were stable under 2–6°C. Stability was established if the change in concentration during the observation period was less than ± 5%.

Ruggedness

The validation results by the different analysts using different columns of the same dimensions (from same vendor but from different lots) meet the acceptance criteria. Around 10 different untreated rats were used just for the plasma collection. The quality control standards prepared from these different blank plasma samples were analyzed. No degradation of the peak and/or the effect of the endogenous material were seen.

Pharmacokinetics

After injecting β-lactamase via intravenous route, the blood samples were collected from the tail vein at different time points. These samples were processed as described earlier and analyzed by the presently developed HPLC method. Figure 5 shows the plasma concentration versus time profile of β-lactamase after intravenous administration to the rats. The hyperbolic shape implied that the in vivo process followed one-compartment first-order kinetics. The linear regression analysis of this semi-loga-

Table I. β-Lactamase HPLC Assay Precision and Accuracy (*n* = 9)

Conc. added (mU/mL)	Conc. obtained (mU/mL)	CV (%)	RE (%)	Recovery (%)
0.315	0.336	6.24	6.6	106.7
3.15	3.24	2.76	2.8	102.3
9.45	9.59	0.57	1.6	101.5

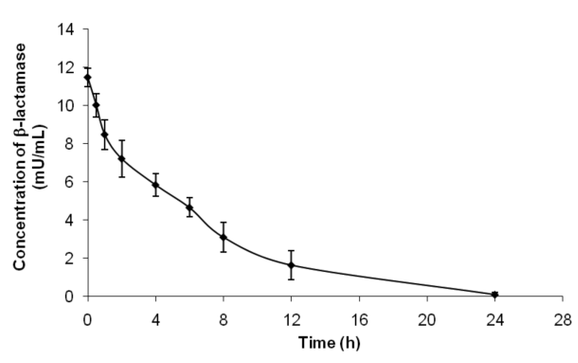

Figure 5. Plasma concentration of β-lactamase after intravenous injection to the rats (Mean ± SD, *n* = 6). Each rat was given β-lactamase solution (200 µL) in PBS through the tail vein. Blood samples were taken from the tail vein at different time points and analyzed by HPLC.

rithmic plot gave r^2 value of 0.97 (± 0.016). Thus it is concluded that the disposition of the drug after intravenous administration follows one-compartment 1st-order kinetics. The pharmacokinetic (PK) parameters such as *AUC, Cl,* $t_{1/2}$, V_d, and K_e were also calculated and are presented in Table II. The elimination half-life is about 4 h. Generally the entire drug is eliminated in 5–6 half-lives, which means that it takes at least 20–24 h for the β-lactamase to get completely eliminated out of the body. The value of Cp_0 was recorded as the *y*-intercept of the concentration-time line by extrapolation of the plasma concentration line to time zero. The volume of distribution (V_d) was then calculated by $Dose/Cp_0$. The calculated V_d is 22 mL in the present case. The volume of distribution is the volume of plasma that would be necessary to account for the total amount of drug in the body. The plasma volume for the rats used in our studies is in the range of 22–25 mL (26). The apparent volume of distribution (V_d = 22 mL) shows that β-lactamases are only distributed in blood, showing a poor access from the infection site (tissues) to blood. Thus it seems reasonable to assume that a large protein molecule like β-lactamase would mainly stay inside the blood circulation after intravenous injection. One-compartment model is based on the assumption of a very fast distribution. Therefore, the Cp_0 obtained by one-compartment model is usually smaller than the actual value, which in turn would lead to a higher V_d. The more accurate method for V_d calculation would be a continuous intravenous infusion until the distribution reaches equilibrium.

Conclusion

The present fluorimetric HPLC method meets all the acceptance criteria and was sensitive and reproducible enough for the acceptable pharmacokinetic study of β-lactamase in plasma. A good linearity of the standard curves with reproducible slopes was observed with this method. The LOQ (up to 10 ng/mL) enabled a full description of the rat intravenous pharmacokinetics of β-lactamase. The assay described here demonstrates that β-lactamase can be detected accurately in the plasma, allowing its pharmacokinetic profile to be elucidated directly. The disposition of β-lactamase follows one-compartment first-order kinetics after intravenous administration to the rats with a half-life of 4 h. The high-throughput analysis achieved using HPLC will make this assay particularly applicable during pharmacokinetic investigations in clinical samples.

Table II. Pharmacokinetic Parameters of β-Lactamase in Rats After Intravenous Administration (*n* = 6)

PK parameter	Mean ± SD
Plasma conc. at the time zero (C_{p0}) (mU/mL)	11.47 ± 0.48
Area under the curve (AUC) (mU.h/mL)	79.4 ± 12.9
Elimination rate constant (Ke) (h^{-1})	0.17 ± 0.02
Clearance Cl (mL/h)	3.63 ± 0.47
Elimination half-life ($t_{1/2}$) (h)	4.12 ± 0.5
Volume of distribution (mL)	22.0 ± 0.9

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