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Development and validation of a LC–MS/MS method for determination of bivalirudin in human plasma: Application to a clinical pharmacokinetic study

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ABSTRACT

A simple, sensitive and rapid LC–MS/MS method has been developed and validated for the identification and quantification of bivalirudin in human plasma using nafarelin as the internal standard. Following protein precipitation with methanol, the analytes were separated on a C_{18} column interfaced with a triple-quadrupole tandem mass spectrometer using positive electrospray ionization. Quantification of bivalirudin was conducted by multiple reaction monitoring (MRM) of the transitions of m/z 1091.4 → (356.4 + 227.4) for bivalirudin and m/z 662.4 → 328.5 for IS. The lower limit of quantification was 1.25 ng/ml, and the assay exhibited a linear range of 1.25–500 ng/ml. The developed assay method was successfully applied to a pharmacokinetic (PK) study in healthy volunteers after intravenous administration of bivalirudin.

1. Introduction

Bivalirudin is a synthetic 20 amino acid peptide analogue of hirudin [1]. Being a direct thrombin inhibitor that binds specifically and reversibly to both fibrin-bound and unbound thrombin, bivalirudin has been used as a new class of anticoagulant for patients presenting with acute coronary syndromes (ACSs) or patients undergoing percutaneous coronary intervention (PCI) [2].

As compared to the comprehensive research on the pharmacological activities of bivalirudin, only a few studies regarding the pharmacokinetic profiles and analytical methods have been done. In these studies [3–5], the authors reported the clinical pharmacokinetic and pharmacodynamic characteristics of bivalirudin, but did not describe the analytical methods. In 2004, Farthing et al. [6] reported a liquid chromatography coupled with fluorescence detector (LC/FD) method to determine bivalirudin in human plasma, by using solid phase extraction (SPE) and o-phthalaldehyde (OPA) derivatization, providing a LLOQ of 3 μg/ml in plasma. Robson et al. [7] developed a liquid chromatographic/tandem mass spectrometric (LC/MS/MS) assay employing an SPE procedure for clinical studies with a linear range for plasma of 0.5–25 μg/ml and LLOQ over 500 ng/ml. The two methods utilizing SPE are expensive and time-consuming.

In the present work, we developed a more sensitive LC/MS/MS method for the determination of bivalirudin with an LLOQ of 1.25 ng/ml. A simple and inexpensive one-step protein precipitation instead of SPE [6–7] was used for plasma sample preparation. After validation, this assay was successfully applied to determine plasma concentrations of bivalirudin after a single intravenous bolus administration of dose 0.75 mg/kg to nine healthy Chinese volunteers.

2. Experimental

2.1. Reagents and chemicals

Bivalirudin (D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu, measured peptide content 101.9%, molecular weight 2180.29 Da) was provided by Yuanshen Pharmaceutical Co., Ltd. (Hebei, China). Nafarelin (Pyr-His-Trp-Ser-Tyr-D-2-Nal-Leu-Arg-Pro-Gly-NH_2, measured peptide content 98.3%, molecular weight 1322.48 Da) for use as the internal standard (IS) was obtained from Nuotai Pharmaceutical Co., Ltd. (Zhejiang, China). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Ammonium acetate, Formic acid and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). The water used was purified on a Milli-Q water purification system (Millipore, Milford, MA, USA).

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2.2. Instrumentation

An Agilent 1200 system (Agilent, Waldbronn, Germany) consisting of a vacuum degasser, a binary pump, an autosampler and a column compartment was used for solvent and sample delivery. An AB MDS Sciex (Concord, Ontario, Canada) API 3200 triple-quadrupole mass spectrometer equipped with a TurboIonSpray ionization (ESI) source was used for mass analysis and detection. A 10-port switching valve (Rheodyne, Cotati, CA, USA) was used to direct HPLC eluate to a waste container in the first 4.0 min of the chromatographic run and afterwards to the ionization source. Data acquisition was performed with Analyst 1.4.2 software (AB MDS Sciex).

2.3. Chromatographic separation

The chromatographic separations were achieved using a Zorbax SB-C_{18} (150 mm × 2.1 mm i.d., 5 μm) column with a Security Guard (12.5 mm × 2.1 mm i.d., 5 μm) C_{18} column (Agilent, Wilmington, DE, USA). The following gradient was used in HPLC determinations: 0–1.5 min, B 50–58%; 1.5–1.6 min, B 58–80%; 1.6–5.5 min, B 80%; 5.5–5.6 min, B 80–50%; 5.6–7 min, B 50%, where A was 0.1% formic acid in water with 25 mM ammonium acetate and B was 0.1% formic acid in methanol. The flow rate of the mobile phase and the column oven temperature were set at 0.3 ml/min and 30 °C, respectively.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode. The tuning parameters were optimized for bivalirudin and the IS by infusing a solution containing 500 ng/ml of both analytes at a flow rate of 5 μl/min into the mobile phase (0.3 ml/min) using a post-column 'T' connection. The curtain gas was set at 15 psi, nebulizer and TurboIonSpray gases (nitrogen) were at 35 and 40 psi. The optimized TurboIonSpray voltage and temperature were set at 5500 V and 400 °C, respectively. For collision-induced dissociation (CID), nitrogen was used as the collision gas at a back-pressure of approximately 5 psi. The declustering potential (DP) was set at 77, 46 V and the collision energy (CE) at 71, 30 V for bivalirudin and the IS, respectively. The entrance potential (EP) was set at 8 V and the collision cell exit potential (CXP) at 4 V. Quantitation was performed using MRM of the transitions of m/z 1091.4 → (356.4 + 227.4) for bivalirudin and m/z 662.4 → 328.5 for the IS. The mass spectrometer was operated at a unit resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3 with a dwell time of 400 ms in each transition.

2.5. Preparation of standard and quality control solutions

Stock solutions were prepared by separately dissolving the accurately weighed reference substance of bivalirudin and the IS in 60% methanol containing 0.05% TFA to yield a concentration of 100 μg/ml, respectively. Bivalirudin working solutions (0.025, 0.06, 0.1, 0.25, 0.5, 1, 1.5, 2.5, and 10 μg/ml) and the IS working solution (100 ng/ml) were prepared by diluting stock solutions with 60% methanol containing 0.05% TFA.

Plasma calibration standards of bivalirudin were prepared by adding 10 μl working solution into 200 μl of drug-free human plasma, yielding final bivalirudin concentrations of 1.25, 3, 5, 12.5, 25, 50, 75, 125, and 500 ng/ml. Quality control (QC) samples were prepared in the same way as calibration. QC sample concentrations were 3, 75 and 500 ng/ml.

All the solutions were stored at −20 °C. For each batch of unknown samples to be analyzed, the appropriate standard and QC solutions were prepared and processed through the plasma sample preparation procedure in parallel with the unknown samples.

2.6. Sample preparation

A 200 μl volume of blank plasma, calibration standards, QC samples and plasma samples, spiked with IS working solution, were pre-treated by protein precipitation with methanol (800 μl). After centrifugation at 14,000 rpm for 10 min at 4 °C, a 900 μl aliquot of the clear supernatant was transferred to another tube and was evaporated to dryness on a Vacuum Centrifugal Concentrator (Thermo, Milford, MA, USA) at 43 °C. The residue was dissolved in 100 μl of the initial mobile phase, vortex-mixed for 30 s, and centrifuged at 14000 rpm for 10 min. The supernatant (70 μl) was pipetted to an autosampler vial, and 20 μl was introduced into the LC/MS/MS system for analysis.

2.7. Method validation

Linearity was analyzed by the weighted regression method ($1/x^2$) of peak area ratios of bivalirudin to IS versus actual concentrations. The calibration curves ($n = 5$) were prepared by spiking blank human plasma with standard solution of bivalirudin. The LLOQ was the smallest analytical concentration at which the precision expressed by the relative standard deviation (RSD) was lower than 20% and the accuracy evaluated by the deviation of the mean from the true value (Dev) was lower than 20%.

QC samples at three concentration levels (3, 75 and 500 ng/ml) were analyzed to assess the accuracy and precision of the method. The assays were performed on 3 separate days, and on each day six replicates of each analyte at each concentration level were analyzed together with an independently prepared calibration curve. The assay accuracy was calculated as (observed concentration − spiked concentration)/(spiked concentration) × 100%. The assay precision for each QC level was determined as RSD of the measured concentrations. The intra- and inter-day precisions were below 15%, and the accuracy was within ±15%.

For the determination of recovery, blank human plasma was processed according to the sample preparation procedure described above. The supernatant was evaporated to dryness, and dry extracts were reconstituted in the initial mobile phase with addition of appropriate standards at concentrations corresponding to the final concentration of the pre-treated plasma samples. These spike-after-extraction samples represented 100% recovery. The extraction recoveries for bivalirudin were determined by comparing the mean peak areas of six extracted low, medium and high QC samples to mean peak areas of six spike-after-extract samples at the same concentrations. The recovery of the IS was also evaluated by comparing the mean peak areas of six extracted medium QC samples to mean peak areas of six reference solutions spiked in extracted plasma samples of the same concentrations.

To evaluate the matrix effect in the experiment, chromatographic peak areas of each analyte from the spike-after-extraction samples, at low, medium and high concentration levels, were compared to those for the clean standard solutions at the same concentrations. Matrix effects for the IS were also investigated.

The stability of bivalirudin in human plasma was also evaluated by analyzing replicates ($n = 3$) of spiked plasma samples (3, 75 and 500 ng/ml), which were exposed to different conditions (time and temperature). These results were compared with those obtained for freshly prepared plasma samples. The short-term stability was determined after the exposure of the spiked samples at 25 °C for 2 h, and the ready-to-inject samples (after protein precipitation) to the autosampler rack (25 °C) for 24 h. The long-term stability was assessed after storage of the standard spiked plasma samples at −20 °C for 45 days. The freeze/thaw stability was evaluated after

three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days.

2.8. Application to a PK study

The LC/MS/MS procedure developed here was used to investigate the plasma profiles of bivalirudin after intravenous administration of 0.75 mg/kg bivalirudin. Nine (5 male and 4 female) Chinese healthy volunteers (aged 20–28, body weight 58.1 ± 6.7 kg) took part in the study after a thorough medical, biochemical and physical examination. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee at The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine. Each volunteer was injected a single dose of 0.75 mg/kg bivalirudin intravenously over a period of 30 s. Venous blood samples of about 1 ml were collected in heparin-containing tubes before drug administration and at 5, 10, 15, 30, 45, 60, 90, 120, 180, and 300 min after the end of the injection. Plasma was separated by centrifugation of treated blood at 4000 rpm for 5 min and stored at −20 °C until analysis. The plasma samples were analyzed within 1 month of storage.

The plasma concentration of bivalirudin versus time data was subjected to a non-compartmental analysis using the computer program Drug and Statistics 1.0 (DAS 1.0) (Medical College of Wannan, China). The elimination rate constant (k) was calculated as the slope of the linear regression fit of the logarithm scale plasma concentrations versus time data for the last four measurable points. Apparent elimination half-life ($t_{1/2}$) was obtained as 0.693/k. The area under the plasma concentration–time curve (AUC) was calculated according to the linear trapezoidal rule to the last measurable point (AUC_{0-t}) or to infinity ($AUC_{0-\infty}$) by $AUC_{0-t} + C_t/k$, where C_t is the last measurable drug concentration. The mean residence time (MRT) was obtained by dividing the area under the first moment-time curve ($AUMC_{0-\infty}$) by the area under the curve ($AUC_{0-\infty}$). The total body clearance (CL) was calculated as dose/$AUC_{0-\infty}$ and apparent volume of distribution (V) as dose/C_0.

3. Results and discussion

3.1. Optimization of the mass spectrometric detection

The $[M+2H]^{2+}$ ions at m/z 1091.4 and 662.4 were used as the precursor ions of bivalirudin and nafarelin, respectively. The product ion mass spectrum of bivalirudin shown in Fig. 1(a) yielded several ions at m/z 650.9, 356.4, 227.4. According to the accepted nomenclature for fragment ions, which was first proposed by Roepstorff and Fohlman [8] and subsequently modified by Johnson et al. [9], the three ions correspond to: 650.9 $[y_5]^+$, 356.4 $[a_3\text{-}NH_3]^+$, 227.4 $[a_4\text{-}NH_3]^{2+}$. The transitions m/z 1091.4 → 356.4 and 1091.4 → 227.4 were chosen for the quantification studies, since they exhibited the highest sensitivity, upon optimization of parameters such as DP and CE. In this study, nafarelin was chosen as IS since it is a peptide that has similar extraction recovery and retention time with the analyte, it was shown to be stable under the described experimental conditions, and it did not interfere with the analysis of bivalirudin. The product ion mass spectrum of nafarelin was shown in Fig. 1(b). The MRM transition of m/z 662.4 → 328.5 was optimized for nafarelin detection, and the ion 328.5 corresponds to $[a_5]^{2+}$.

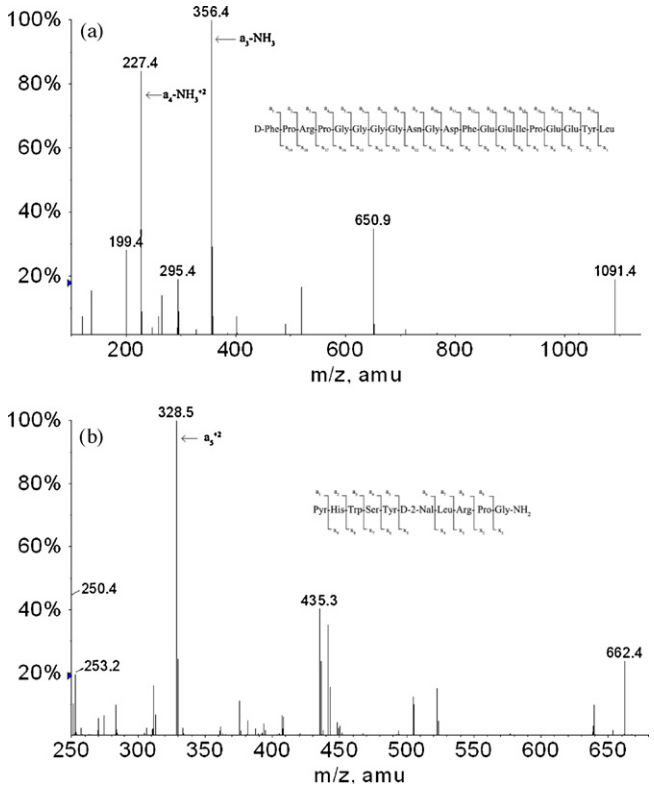

Fig. 1. (a) Product ion spectra of bivalirudin (b) product ion spectra of nafarelin.

3.2. Optimization of the chromatographic condition

The chromatographic conditions were optimized by varying mobile phase, gradient elution type, flow rate and type of columns. LC was run initially on a Zorbax SB-C_{18} (150 mm × 4.6 mm i.d., 5 μm) column. However, by changing to the described Zorbax SB-C_{18} (150 mm × 2.1 mm i.d., 5 μm) column, peak sharpness was achieved, with enhanced signal intensity and ability to separate from possible interfering components. It was also found that formic acid and ammonium acetate in the mobile phase could shorten the chromatographic time and improve the peak shape of bivalirudin and the IS, but it had no profound effect on the sensitivity for the analyte under the chromatographic conditions.

3.3. Sample preparation

Sample preparation is a critical step for accurate and reliable LC/MS/MS assays. Several extraction procedures were tested, including SPE and the protein precipitation method. Initially, we used a SPE method available commercially, the Oasis® C_{18} HLB SPE plate (Waters, Milford, MA, USA). However, the recovery of bivalirudin using SPE was low, at approximately 50%. Alternatively, we employed the protein precipitation method. Extraction of bivalirudin with methanol was shown to be an adequate and rather low cost method for the extraction of bivalirudin (recoveries over 80%).

Table 1
Precision and accuracy for the analysis of bivalirudin in human plasma (n = 3 days, six replicates per day).

Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Inter-day RSD (%)	Intra-day RSD (%)	Accuracy percent error (%)
3.00	2.92 ± 0.31	10.4	10.6	−2.7
75.0	74.4 ± 6.08	7.7	8.2	−0.8
500	484 ± 37.1	5.7	7.7	−3.2

3.4. Selectivity and matrix effect

Selectivity was assessed by comparing chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with bivalirudin (75 ng/ml) and IS (25 ng/ml), and a plasma sample from a healthy volunteer 0.5 h after an intravenous administration. No significant interference from endogenous substances with bivalirudin or nafarelin was detected. Typical retention times for bivalirudin and nafarelin were 5.5 and 5.7 min, respectively.

Matrix effects on recovery of blank plasma samples spiked after the sample preparation with 3, 75 and 500 ng/ml of bivalirudin were found to be within the acceptable limits (95–105%). The same evaluation was performed on the IS, and no significant peak area differences were observed. Thus, ion suppression or enhancement from plasma matrix was negligible for this method.

3.5. Linearity and LLOQ

Good linearity was obtained in the range of 1.25–500 ng/ml in human plasma. The mean linear regression equation of the calibration curves generated during the validation was: $y = 3.12(\pm 1.68) \times 10^{-2} + 1.89(\pm 0.12) \times 10^{-2}x$, where y represents the peak area ratio of bivalirudin to IS, and x is plasma concentration of bivalirudin in ng/ml. The correlation coefficients of the weighted calibration curves range from 0.996 to 0.999. Good linearity was obtained in the validated concentration range.

The present method offered an LLOQ of 1.25 ng/ml for bivalirudin using only 0.2 ml plasma. The RSD ($n = 6$) was 12.8% and accuracy was 5.6% at this concentration. Compared with the method reported using MS detector [3], a higher sensitivity for bivalirudin was obtained in our study and made this method more advantageous to measure the trace concentration of bivalirudin in plasma.

3.6. Precision and accuracy

Table 1 summarizes the intra- and inter-day precision and accuracy for bivalirudin, evaluated by assaying the QC samples. In this assay, the inter-day precision was within 5.7–10.4% and the intra-day precision was within 7.7–10.6% or less for each QC level. The accuracy was within ±5.1%. These results demonstrated that the values were within the acceptable range and the method was sufficiently accurate and precise.

3.7. Extraction recovery and analyte stability

Mean extraction recoveries of bivalirudin at 3, 75 and 500 ng/ml were 84.8 ± 4.7%, 93.2 ± 1.7% and 91.5 ± 8.8%, respectively (mean ± SD, $n = 6$). Mean recovery of the internal standard

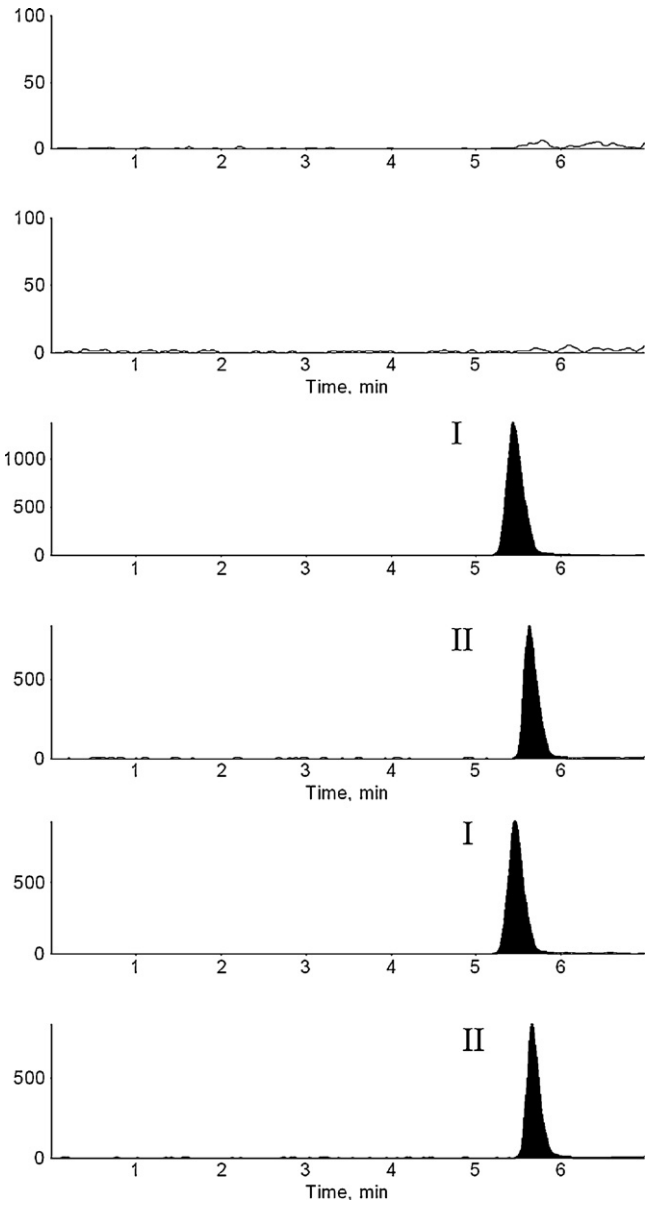

Fig. 2. Representative MRM chromatograms for bivalirudin (I) and IS (nafarelin, II) in human plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with bivalirudin (75 ng/ml) and IS (25 ng/ml); (C) a plasma sample from a volunteer 0.5 h after intravenous administration of 0.75 mg/kg bivalirudin.

Table 2
Stability of bivalirudin under a variety of storage conditions ($n = 3$).

Storage conditions	Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	Percentage recovery (%)	RSD (%)
Short-term (2 h at 25 °C)	3.00	2.97	99.0	11.7
	75.0	71.7	95.6	4.4
	500	474	94.8	1.4
Long-term (45 days at −20 °C)	3.00	2.68	89.3	8.5
	75.0	66.2	88.3	3.1
	500	484.3	96.9	0.7
Three freeze/thaw cycles	3.00	2.96	98.7	2.6
	75.0	66.7	88.9	2.4
	500	478	95.6	1.3
Autosampler for 24 h (25 °C)	3.00	3.13	104.3	9.1
	75.0	66.7	88.9	0.3
	500	477	95.4	1.1

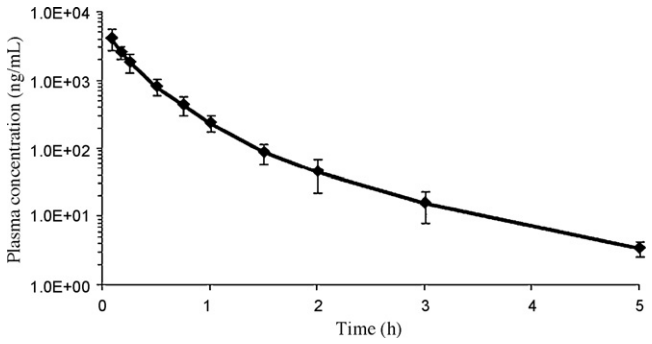

Fig. 3. The mean plasma concentration–time curves of bivalirudin after intravenous administration of 0.75 mg/kg to healthy volunteers ($n = 9$, mean ± SD).

Table 3
The main pharmacokinetic parameters of bivalirudin after intravenous administration of 0.75 mg/kg to nine healthy volunteers.

Parameter	Mean ± SD
k (h^{-1})	2.24 ± 0.50
$t_{1/2}$ (h)	0.32 ± 0.07
$AUC_{(0-5h)}$ (μg h/l)	1415.98 ± 273.44
$AUC_{(0-\infty)}$ (μg h/l)	1421.15 ± 274.12
V (l/kg)	0.1966 ± 0.0693
CL (l/kg/h)	0.5473 ± 0.1161
MRT (h)	0.47 ± 0.10

was 88.6 ± 1.2% (mean ± SD, $n = 6$). The RSDs for all recoveries were less than 15% throughout the entire concentration ranges, indicating solid assay consistency.

The stability of bivalirudin was investigated at different concentrations under a variety of storage and process conditions. The results are summarized in Table 2. This data demonstrated that bivalirudin was stable in plasma when stored at 25 °C for 2 h, −20 °C for 45 days, after three freeze/thaw cycles (from −20 to 25 °C). The analyte in the final extract was also proved to be stable for over 24 h in the autosampler.

3.8. Application of the method to PK studies in healthy volunteers

We developed the present method with the LLOQ down to 1.25 ng/ml to satisfy the demand of evaluating pharmacokinetics of the drug. Using this analytical method, we were able to measure the concentration of bivalirudin up to 5 h for all subjects. Fig. 3 shows the profile of the mean bivalirudin plasma concentration versus time. Meanwhile, the major PK parameters of bivalirudin are listed in Table 3.

The pharmacokinetics are characterized by a small volume of distribution ($V = 0.197$ l/kg), rapid plasma clearance (CL = 0.55 l/h/kg) and an elimination half-life $t_{1/2}$ of about 0.32 h. It is in agreement with the published pharmacokinetic studies [3–5].

4. Conclusion

An LC/MS/MS assay for bivalirudin in human plasma was developed and validated with respect to linearity, precision and accuracy, and analysis of real samples was demonstrated. The method needed a simple protein precipitation procedure followed by the analysis of a total running time of 7.0 min per sample with the LLOQ of 1.25 ng/ml. The assay was applied to characterize the pharmacokinetics of bivalirudin in healthy volunteers after intravenous administration of 0.75 mg/kg.

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