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Development of a liquid chromatography/tandem mass spectrometry assay for the determination of bestatin in rat plasma and its application to a pharmacokinetic study

Chen Wang [a,b], Guorong Fan [a,b,*], Mei Lin [c,**], Yi Chen [a,b], Weiquan Zhao [a,b], Yutian Wu [a,b]

[a] *Department of Pharmaceutical Analysis, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China*
[b] *Shanghai Key Laboratory for Pharmaceutical Metabolite Research, No. 325 Guohe Road, Shanghai 200433, PR China*
[c] *Shanghai Institute for Drug Control, No. 615 Liuzhou Road, Shanghai 200233, PR China*

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Abstract

Bestatin is a low molecular weight aminopeptidase inhibitor originally isolated from culture filtrates of *Streptomyces olivoreticuli*. We have developed a sensitive, specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the quantitative determination of bestatin in rat plasma using granisetron as the internal standard. The analyte and internal standard were isolated from 50 μL plasma samples by solid phase extraction (SPE). Reverse-phase HPLC separation was accomplished on a Lichrospher C18 column (4.6 mm × 50 mm, 5 μm) with a mobile phase composed of methanol–water–formic acid (70:30:0.5, v/v/v) at a flow rate of 0.8 mL/min. The method had a chromatographic total run time of 3 min. A Varian 1200L electrospray tandem mass spectrometer equipped with an electrospray ionization source was operated in selected reaction monitoring (SRM) mode with the precursor-to-product ion transitions m/z 309.2 → 120.0 (bestatin) and 313.4 → 138.0 (granisetron) used for quantitation. The method was sensitive with a lower limit of quantitation (LLOQ) of 5 ng/mL, with good linearity ($r^2 \geq 0.999$) over the linear range of 5–2000 ng/mL. All the validation data, such as accuracy, precision, and inter-day repeatability, were within the required limits. The method was successfully applied to pharmacokinetic study of bestatin in rats.

Keywords: Bestatin; LC–MS/MS; Pharmacokinetics

1. Introduction

Bestatin (2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl-S-leucine, is an aminopeptidase B and leucine aminopeptidase inhibitor originally isolated from culture filtrates of *Streptomyces olivoreticuli* [1]. This microbial product has shown various beneficial immunological activity, including enhancement of immunological responses that have been weakened in the presence of cancer [2–4], enhancement T-cell as well as macrophage mediated immunoreactions [5], promotion of the host's antitumor activity [6,7], and resistance to infection [8,9]. It can also enhance the G- and GM-CSF-induced colony formation to modulate the proliferation and differentiation of human bone marrow cells [10,11]. Because of these significant therapeutic activities, bestatin has been studied as a therapeutic drug for cancer, resistant infection, muscular dystrophy [12], myelodysplastic syndrome and chronic leukemia [13].

To meet the demands for the pharmacokinetic study of bestatin, a rapid, selective and robust analytical method is highly desirable. Previous investigation of bestatin in biological samples including HPLC [14–16], gas chromatographic–mass spectrometry [17], however, these methods do not meet modern drug development needs with respect to an efficient extraction procedure, shorter run time and sensitivity, as they require prior derivatization and laborious extraction procedure. Liquid

* Corresponding author at: Second Military Medical University School of Pharmacy, 325 Guohe Road, Shanghai 200433, China. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
** Corresponding author. Tel.: +86 21 6470 5560; fax: +86 21 6482 9580.
E-mail addresses: Guorfan@yahoo.com.cn (G. Fan), linmeish@yahoo.com.cn (M. Lin).

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chromatography–tandem mass spectrometry (LC–MS/MS) has emerged as a sensitive and selective analytical method in drug analysis. We herein describe a simple, sensitive and high throughput method based on solid phase extraction (SPE) and LC–MS/MS for routine measurement of bestatin using granisetron as the IS in rat plasma in support of pharmacokinetic study. To the best of our knowledge, studies on LC–MS/MS in the analysis of bestatin in biological sample have not yet been reported.

2. Experimental

2.1. Chemicals and reagents

Bestatin and granisetron hydrochloride (IS) were obtained from National institute for the control of pharmaceutical and biological products (Beijing, PR China). The purities of bestatin and granisetron hydrochloride were >99.5%. Formic acid (chromatographic grade) was purchased from Tedia (Fairfield, USA). Methanol (chromatographic grade) was purchased from Merck (Darmstadt, German). Deionized (18 MΩ/cm) water was generated in-house using a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. LC–MS/MS Instrumentation

A Varian HPLC–MS/MS system (Palo Alto, CA, USA) consisted of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200L triple quadrupole mass spectrometer equipped with an electrospray ionization source. Varian MS workstation version 6.3 software was used for data acquisition and processing.

2.3. Liquid chromatographic conditions

The chromatographic separation was performed on a Lichrospher C18 column (4.6 mm × 50 mm ID, 5 μm particle size, Hanbon Science & Technology Co. Ltd., Jiangsu, PR China) thermostated at 30 °C with the mobile phase composed of methanol–water–formic acid (70:30:0.5, v/v/v) at a flow rate of 0.8 mL/min. Before use, the mobile phase was filtered through a 0.45 μm nylon membrane filter. The injection volume was 10 μL and the analysis time was 3 min per sample.

2.4. Mass spectrometer conditions

The HPLC eluant was split 1:4 to flow 160 μL into the mass spectrometer. The ESI–MS spectrometer was operated in the positive ion mode. The electrospray capillary voltage was set at 35 V. Nitrogen was used as a drying gas for solvent evaporation. The API housing and drying gas temperatures were kept at 50 °C and 380 °C. Protonated analyte molecules were subjected to collision induced dissociation using argon as the collision gas to yield product ions for each analyte. The collision energy was 14 eV for bestatin and 16 eV for the IS. The scan time was 1 s and the detector multiplier voltage was set to 1330 V. Selected reaction monitoring (SRM) of the precursor-product ion transitions *m/z* 309.2 → 120.0 for analyte and 313.4 → 138.0 for IS was used for quantitation. Product ion mass spectra for analyte and IS are shown in Fig. 1.

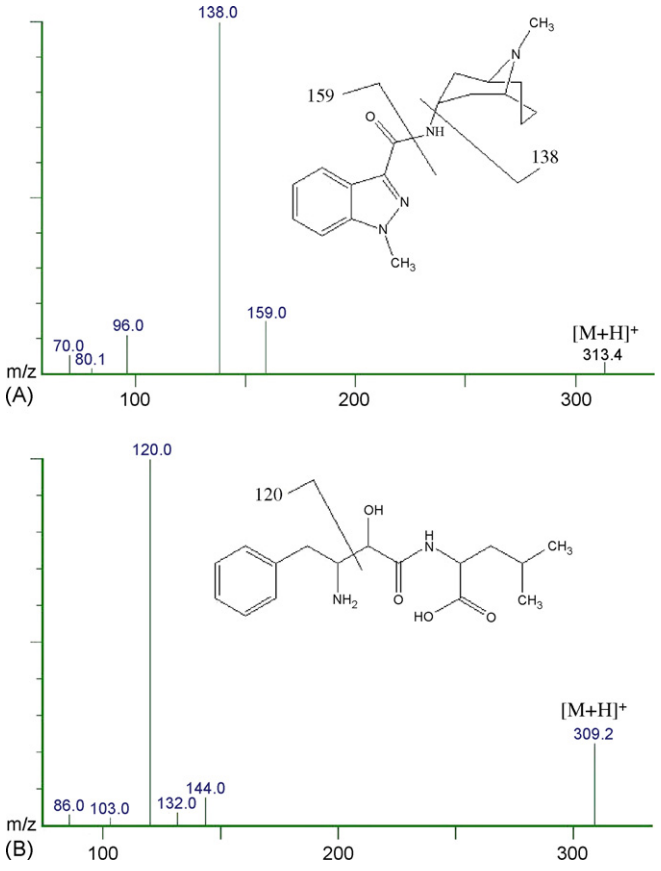

Fig. 1. Chemical structures and product ion spectra of $[M+H]^+$ of bestatin (A) and granisetron (B).

2.5. Preparation of standard and quality control (QC) samples

Primary stock solutions of bestatin were prepared by dissolving the accurately weighed bestatin in methanol to yield a final concentration of 1 mg/mL. The solutions were sonicated for 5 min to ensure complete dissolution. Following sonication, the solutions were allowed to equilibrate to room temperature after which they were diluted. Working standards of bestatin were prepared from individual aliquots of 1 mg/mL stock solution at 0.05–20 μg/mL using H_2O: methanol (55:45, v/v) as the diluent. The stock standard solution of IS was prepared by dissolving appropriate amounts of granisetron hydrochloride in methanol to give a final base concentration of 1 mg/mL. A 4 μg/mL internal standard working solution was obtained by diluting the stock solution of granisetron with H_2O: methanol (55:45, v/v). All the solutions were stored at 4 °C and brought to room temperature before use. Plasma standards (5, 10, 20, 50, 100, 200, 500, 1000, 2000 ng/mL) were prepared daily by spiking 5 μL of each working standard (0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 μg/mL) into 50 μL of rat plasma. These standards were used to construct calibration curves for the

quantitation of bestatin at plasma concentrations ranging from 5 to 2000 ng/mL. QC stock solutions (0.3 mg/mL) were prepared separately by dissolving the accurately weighed bestatin. QC samples of 15, 150 and 1500 ng/ml were prepared by spiking 250 μL of diluted QC stock solutions (0.3, 3, 30 μg/mL) into 4750 μL of rat plasma, and were aliquoted into 200 μL non-sterile eppendorf tubes and stored at −20 °C until analysis.

2.6. Extraction procedure

The samples of rat plasma were taken out from −20 °C freezer and kept at room temperature for thawing. The samples were vortexed adequately before pipetting. Fifty microliter of plasma was transferred into eppendorff micro-tube to which 5 μL of IS working solution (4 μg/mL) was added and vortexed to mix. These samples were loaded on Supelco LC-18 cartridge previously conditioned with 2 ml methanol followed by 1 ml water. The SPE cartridge was washed with 1 ml of methanol: water (5: 95, v/v) and eluted with 1 ml of formic acid: methanol (2: 98, v/v). Extracts were concentrated to dryness at 40 °C under a gentle stream of nitrogen and reconstituted with 100 μL of H_2O: methanol (55:45, v/v). A 10 μL aliquot of the solution was injected into the LC–MS/MS system for analysis.

2.7. Pharmacokinetic study in rats

Six Sprague–Dawley rats (both sexes) weighing 228 ± 8 g were received an oral administration of bestatin at 2.5 mg/kg dissolved in 0.5% carboxy methyl cellulose after an overnight fast. Animal had access to water and food 4 h after drug administration. Through the catheters which had been implanted into the right external jugular vein of adult rats 1 day prior to the experiments, blood samples (0.5 mL) were collected into heparinized tubes before administration and at different time points (0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h) after administration, and physiologic saline (0.5 mL) was administrated to compensate for the blood loss after every blood sample. The plasma was separated from heparinized blood by centrifugation and stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Method development

3.1.1. Selection of internal standard

It is necessary to use an IS to get high accuracy when a mass spectrometer is equipped with HPLC as the detector. Granisetron was adopted in the end because of its similarity of retention action, ionization and extraction efficiency as well as its less endogenous interference at *m/z* 313.4.

3.1.2. Sample pre-treatment

Due to the complex nature of plasma, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS/MS analysis. Liquid–liquid extraction with ethyl acetate and protein precipitation with acetonitrile were evaluated as sample pre-treament techniques in our earlier study. However, these techniques resulted in strong interferences from the sample matrix and low recoveries of both analyte and IS. Solid phase extraction has been demonstrated as an effective sample pre-treatment procedure to remove protein and potential interfering endogenous components in plasma and by changing sobent materials and solvent composition can optimize its selectivity. Therefore, SPE was selected as sample pre-treatment technique.

Different types of SPE columns were evaluated for the extraction, on which the recoveries of bestatin and IS were carefully compared. Finally, Supelco LC-18 columns were chosen for the sample pre-treatment. Different conditioning, washing and elution solvents were tested for the further optimization of the procedure. The recovery of IS was low (<20%) when methanol was used as elution solvent, and an addition of 2% formic acid to methanol showed a beneficial effect for the recovery of IS (69.6%), as the acid modifier can reduce the retention of granisetron, a weak organic base, in the SPE columns, while the recovery of bestatin is similar.

3.1.3. LC–MS/MS optimization

Parameters of MSD were tuned according to the MS signal response of the target compound and the results indicated that the positive mode was much more sensitive than the negative mode. In the positive ESI mode, the analyte and IS formed predominately protonated molecular ions $[M+H]^+$ (*m/z* 309.2 and *m/z* 313.4) in full scan mass spectra. In the product ion spectra, several fragment ions were obtained, but the ion at *m/z* 120.0 and 138.0 were chosen because they displayed much greater intensity than the others in the acquisition of bestatin and IS, respectively.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and the IS, as well as a short run time. Modifiers, such as ammonium acetate and formic acid alone or with combination in different concentrations were added. It was found that a mixture of methanol–water–formic acid (70:30:0.5, v/v/v) could achieve this purpose and was finally adopted as the mobile phase. The percentage of formic acid was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in mass spectrometer. After careful comparison of many columns, a Lichrospher C18 column (4.6 mm × 50 mm, 5 μm) was finally used with a flow rate of 0.8 mL/min to produce good peak shapes and permit a run time of 3 min.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of blank plasma and the spiked plasma. Under the above conditions the retention time of bestatin and IS was 1.2 and 1.3 min, respectively. All plasma lots were found to be free of interferences with the compounds of interest. A representative chromatogram of a control plasma double blank is shown in Fig. 2A.

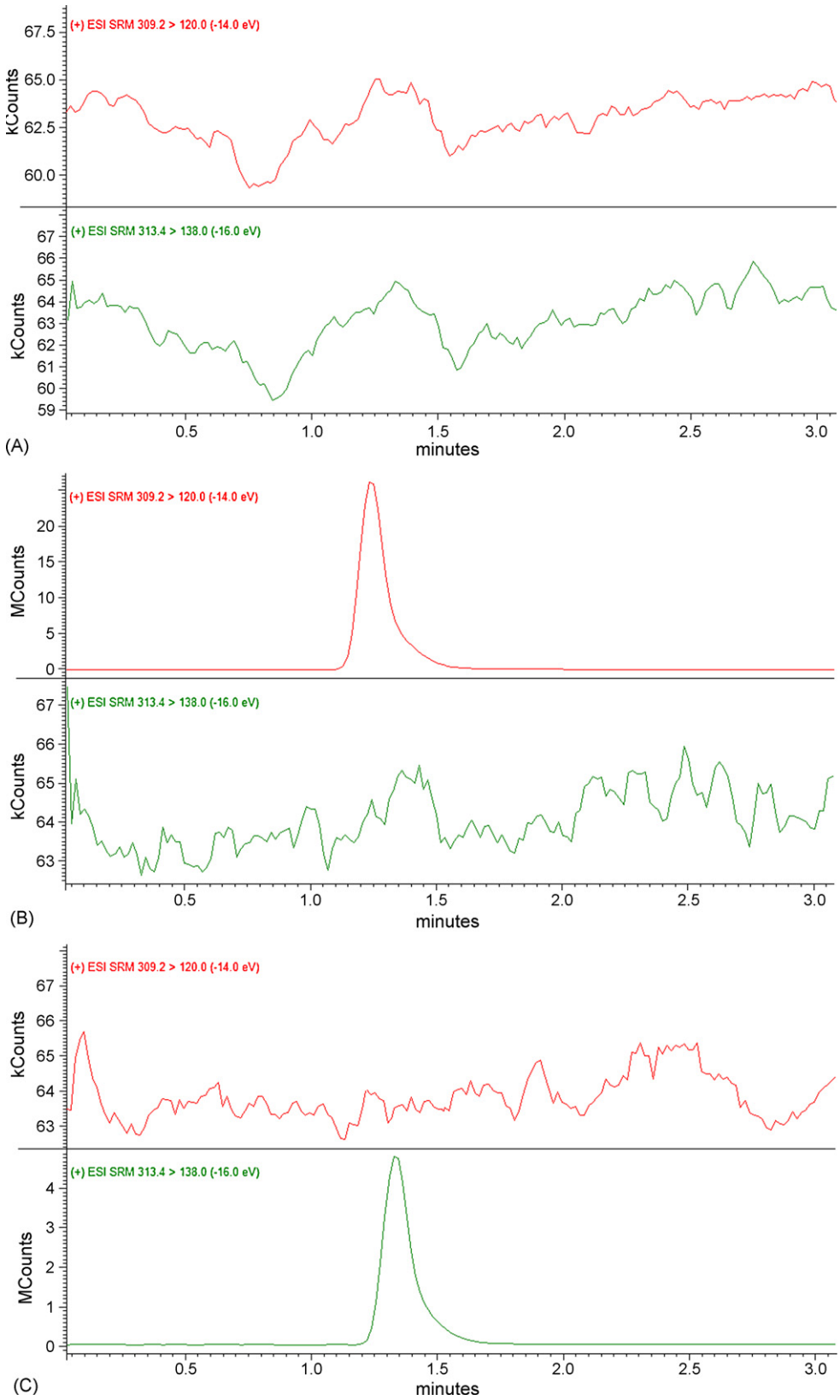

Fig. 2. Representative chromatograms: (A) control plasma double blank; (B) blank plasma spiked with 2000 ng/mL of bestatin; (C) blank plasma spiked with 400 ng/mL of IS; (D) 5 ng/mL plasma standard; (E) plasma sample collected 24 h after an oral administration of bestatin. The measured concentration of bestatin in this sample was 9.4 ng.

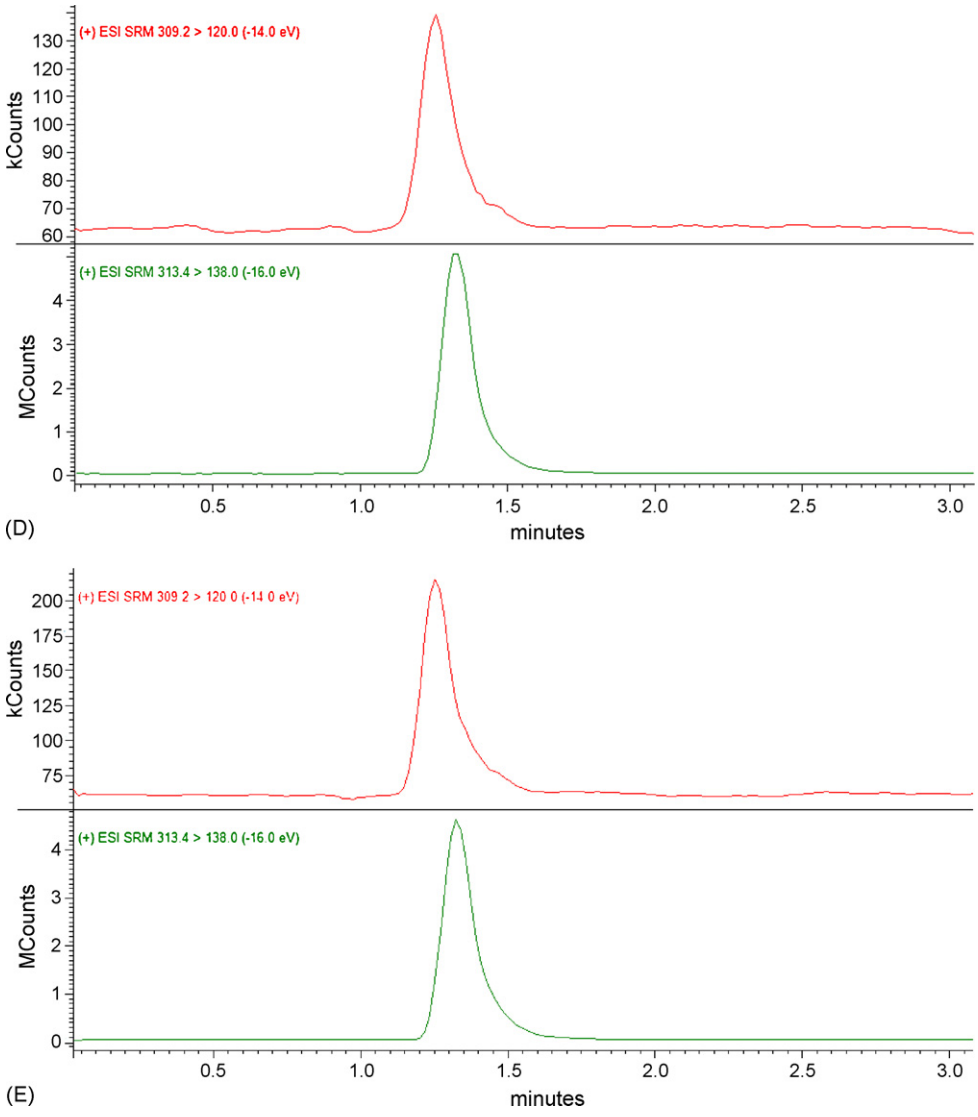

Fig. 2. (*Continued*).

In addition, the HPLC–MS/MS system was evaluated for the presence of "cross-talk" between the channels used for monitoring bestatin and IS. Fig. 2B and C clearly show the absence of any MS/MS response from the analyte into internal standard channel and *vice versa*.

The matrix effects are generally due to the influence of coeluting compounds on the actual analyte ionization process [18]. The effects of the plasma matrix on ionization efficiency were expressed as the ratio of the mean peak area of analytes spiked after extraction from five different lots of plasma (i.e. lots originating from five rats, respectively) to that of the neat standards at different concentrations. A mean ionization suppression of 25.8% (CV = 2.9%), 29.3% (CV = 2.2%), 26.7% (CV = 2.8%), 28.1% (CV = 1.7%) for bestatin (5, 15, 150, 1500 ng/mL) and 21.1% (CV = 1.9%) for granisetron (400 ng/mL) were observed. The low CVs of these experiments indicated the lack of a relative matrix effects. The result for granisetron is not in accordance with the literature. Under almost the same mass spectrometer conditions, using the same type of liquid chromatographic column, except for the different sample pretreatment, a mean ionization enhancement of 27% for granisetron was observed [19,20]. To account for this phenomenon, three different extraction procedures were used to evaluate the relations between the sample pretreatment procedures and matrix effects: an ethyl acetate liquid–liquid extraction, an acetonitrile protein precipitation and a solid phase extraction. The result is shown in Table 1. It is important to state that sample pretreatment is responsible for matrix effect in this assay, which has been reported by D.L. Buhrman et al. in the determination of SR 27417 by LC–MS/MS [21]. The solid phase extraction, which contained the fewest coeluting components, gives the most acceptable result. The similar ionization suppression during ionization in the electrospray source did not affect the ratios of peak area of bestatin and IS that were used for constructing the standard curve.

3.2.2. *Sensitivity and linearity*

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve

Table 1
Matrix effect[a] for bestatin and IS ($n = 5$)

Nominal concentration (ng/mL)	Extraction procedure		
	SPE (%)	PP[b] (%)	LLE[c] (%)
5	74.2	34.4	268.8
15	70.7	33.8	155.7
150	73.3	37.5	108.2
1500	71.9	35.2	103.1
400 (IS)	78.9	36.5	129.1

[a] Matrix effect is expressed as the ratio of the mean peak of the analytes spiked into plasma post-extraction to the mean peak of the neat standards ($\text{area}_{\text{post-extraction}}/\text{area}_{\text{neat}} \times 100\%$).
[b] Protein precipitation with acetonitrile.
[c] Liquid–liquid extraction with ethyl acetate.

that can be quantitated with accuracy within 15% of nominal and precision not exceeding 15% CV, was 5 ng/mL. The reproducibility of LLOQ was determined by examining five LLOQ samples independent from the standard curve, and the accuracy and precision was −7.5% and 10.9%, respectively. A typical chromatogram of an LLOQ sample is shown in Fig. 2D.

Calibration curves were constructed by plotting the peak area ratios (bestatin/IS) of plasma standards versus nominal concentration. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x$ weighing factor, giving a mean linear regression equation for the calibration curve of: $y = 0.002315x + 0.0120$, $r^2 = 0.9997$, where y represents the peak area ratios of bestatin to that of IS, and x represents the plasma concentration of bestatin in ng/mL. Calibration curves of five different lots of plasma were linear in the range of 5–2000 ng/mL with $r^2 \geq 0.999$. The difference between the nominal standard concentration and the back-calculated concentration from the weighed linear regression line was varied from −5.8% to 5.0% for each point on the standard curve (CV varied from 1.5% to 9.3%).

3.2.3. Accuracy and precision

The method showed good accuracy and precision. Table 2 shows a summary of intra- and inter-day accuracy and precision for bestatin from the QC samples, respectively. In this assay, the intra-day precision was less than 5.4%, the inter-day precision was less than 7.1%. The accuracy was from −2.5% to 7.6%.

Table 3
Extraction recovery ($n = 5$)

Nominal concentration (ng/mL)	Peak area[a] (A)	Peak area[b] (B)	Extraction recovery[c] (%) (A/B)
15	1.71×10^6	2.26×10^6	75.7
150	18.41×10^6	23.70×10^6	77.7
1500	174.45×10^6	230.87×10^6	75.6
400 (IS)	40.22×10^6	57.80×10^6	69.6

[a] Standards spiked before extraction.
[b] Standards spiked after extraction.
[c] Extraction recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma pre-extraction (A) to the mean peak area of the analytes spiked into plasma post-extraction (B).

3.2.4. Extraction recovery

To investigate extraction recovery, a set of samples ($n = 5$ at each concentration in unique lots of plasma) was prepared by spiking bestatin into plasma at 15, 150, and 1500 ng/mL. Each of the samples was also spiked with IS at the working concentration of 400 ng/mL. The samples were subsequently processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction with the same concentrations of bestatin and IS as the pre-extraction spiked samples. Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to that of the samples spiked after extraction. The results are indicated in Table 3. Mean extraction recoveries of bestatin at concentrations 15, 150, 1500 ng/mL were 75.7%, 77.7% and 75.6%, respectively, and the extraction recovery of the IS was 69.6%, as shown in Table 3.

3.2.5. Stability

Bench-top stability was investigated to ensure that bestatin was not degraded in plasma samples at room temperature for a time period to cover the sample preparation, and was assessed by exposing the QC samples to ambient laboratory conditions for 10 h. Freeze-thaw stability was assessed over three cycles. QC samples were thawed at room temperature and refrozen at −20 °C over three cycles and assayed. Due to the need for occasional delayed injection or reinjection of extraction samples, the stability of reconstituted samples in autosampler vials was assessed at ambient temperature for over 24 h. The freezer storage stability of bestatin in rat plasma at −20 °C was evaluated by assaying QC samples at beginning and 2 weeks later. All stability QC samples were analyzed in five replicates. The result indicated that bestatin

Table 2
Summary of precision and accuracy from QC samples of bestatin in rat plasma ($n = 5$)

Nominal concentration (ng/mL)	Intra-day			Inter-day		
	Measured concentration (ng/mL) (mean ± SD)	CV (%)	RE[a] (%)	Measured concentration (ng/mL) (mean ± SD)	CV (%)	RE[a] (%)
15	15.6 ± 0.9	5.4	3.8	16.2 ± 1.1	7.1	7.6
150	152.6 ± 4.0	2.6	1.7	147.8 ± 3.4	2.3	−1.5
1500	1462.5 ± 97.5	1.6	−2.5	1488.5 ± 23.1	1.6	−0.8

[a] RE is expressed as [(mean measured concentration)/(nominal concentration) − 1] × 100.

Table 4
Stability of bestatin in rat plasma ($n = 5$)

Sample condition	Nominal concentration (ng/mL)	Measured concentration (ng/mL) (mean ± SD)	CV (%)	RE[a] (%)
Bench top stability[b]				
	15	14.8 ± 1.0	6.5	−1.0
	150	149.8 ± 3.6	2.4	−0.15
	1500	1481.3 ± 28.3	1.9	−1.2
Autosampler stability[c]				
	15	15.3 ± 1.0	6.5	1.7
	150	146.8 ± 4.8	3.3	−2.1
	1500	1492.4 ± 23.5	1.6	−0.5
Freeze-thaw stability[d]				
	15	15.5 ± 0.9	5.6	3.4
	150	146.6 ± 4.8	3.3	−2.3
	1500	1492.8 ± 25.1	1.7	−0.5
2-week storage stability[e]				
	15	15.6 ± 1.1	7.0	4.2
	150	149.7 ± 5.6	3.8	−0.2
	1500	1498.4 ± 27.0	1.8	−0.1

[a] RE is expressed as [(mean measured concentration)/(nominal concentration) − 1] × 100.
[b] Exposed at ambient temperature (25 °C) for 10 h.
[c] Kept at ambient temperature (25 °C) for 24 h.
[d] After three freeze-thaw cycles.
[e] Stored at −20 °C.

had an acceptable stability under those conditions, as shown in Table 4.

3.3. Application of the assay

The method described above was applied to study pharmacokinetics after oral administration of bestatin at 2.5 mg/kg. A representative chromatogram from a post-dose sample is shown in Fig. 2E. The mean plasma concentrations-time profiles of bestatin after an oral administration are shown in Fig. 3. The concentration-time data were analyzed by non-compartmental method and the pharmacokinetic parameters are shown in Table 5. J. Ishida et al. and M. Koyama et al. reported the bestatin reached the C_{max} at 0.5 h and 1–2 h after drug administration in healthy volunteers, respectively [16,17], while in the patients with myelodysplastic syndrome, T. Ueda et al. reported the concentration of bestatin increased gradually up to 7.1 mg/mL at 5 h after an oral administration of 150 mg [15]. F. Abe et al. reported

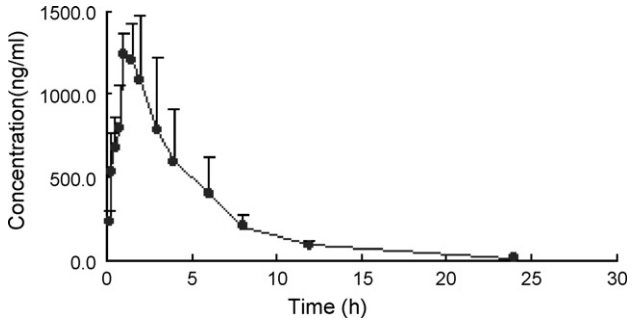

Fig. 3. Mean plasma concentration vs. time after an oral administration of bestatin (2.5 mg/kg).

Table 5
Mean pharmacokinetic parameters after oral administration of bestatin in six rats (2.5 mg/kg)

Pharmacokinetic parameters	Mean ± SD
T_{max}	1.3 ± 0.4
$t_{1/2}$	3.5 ± 0.7
MRT	4.9 ± 0.3
$AUC_{0-\tau}$ (ng h/mL)	$6.2 \times 10^3 \pm 2.1 \times 10^3$
$AUC_{0-\infty}$ (ng h/mL)	$6.3 \times 10^3 \pm 2.1 \times 10^3$

the C_{max} was achieved 0.25 h after oral administration in mice [14]. To the best of our knowledge, studies on pharmacokinetics of bestatin in rats have not yet been reported. In our study, the C_{max} of bestatin of 1.4 μg/mL was obtained at 1.3 h after an oral administration of bestatin at 2.5 mg/kg in rats. Species difference may be one of the main reasons of the pharmacokinetic parameters variation.

4. Conclusion

A sensitive and specific liquid chromatography–tandem mass spectrometric method has been developed for the determination of bestatin in rat plasma. The adequate selectivity, sensitivity, precision and accuracy obtained using small sample volume (50 μL) make it suitable for the purpose of the pharmacokinetic study. Because of the relative short chromatographic run timer and straightforward sample pre-treatment procedure, make the method easy and fast to perform. The method has been successfully applied to the pharmacokinetic study of bestatin in rats.

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