Chromatographia (2012) 75:721–727
DOI 10.1007/s10337-012-2248-y

ORIGINAL

Determination of Boc5 in Rat Plasma by HPLC–MS/MS and Its Application to a Pharmacokinetic Study

Ruixing Li · Huirong Fan · Yuan Gu · Duanyun Si · Wei Liang

Received: 21 February 2012 / Revised: 11 April 2012 / Accepted: 23 April 2012 / Published online: 22 May 2012
© Springer-Verlag 2012

Abstract A simple, robust, and sensitive high-performance liquid chromatography tandem mass spectrometry method was developed and validated for determination of a new nonpeptidic glucose-like peptide-1 receptor (GLP-1R) agonist 1,3-bis(4-((*p*-butoxycarbonyl)amino)benzamido)-2,4-bis(*m*-methoxy-*p*-(thiophene-2-methanoyl)phenyl)-cyclobutane-1,3-dicarboxylic acid (Boc5) in rat plasma using wogonin as internal standard (IS). After a simple protein precipitation with methanol, chromatographic separation was achieved on an Atlantis T3 (100 mm × 4.6 mm, 3.5 μm) analytical column eluted by mobile phase consisting of methanol, acetonitrile, and 20 mM ammonium formate addition to 2% formic acid (39:39:22, *v*/*v*/*v*) at flow rate of 0.6 mL/min for 7 min. The quantitation analysis was performed using multiple reaction monitoring at the transitions of *m*/*z* 1,077.2 → 784.4 for Boc5 and *m*/*z* 285.1 → 270.4 for IS in positive ion mode with electrospray ionization source on an AB SCIEX API 4000 QTRAP mass spectrometer. Good linearity was achieved over the concentration range of 2–2,000 ng/mL with lower limit of quantification at 2 ng/mL. Intra- and interday precisions were less than 7.3 %, and accuracy ranged from −3.6 to 3.0 %. The mean recovery of Boc5 was 93.4–100.2 %, and the matrix effect of Boc5 was 88.0–93.8 %. The validated method was then successfully applied to a pharmacokinetic study of Boc5 in rats.

Keywords HPLC–MS/MS, Boc5 · Nonpeptidic GLP-1R agonists · Pharmacokinetics

Introduction

Glucose-like peptide-1 (GLP-1), a 30-amino-acid peptide hormone, is secreted from the L-cells that are located in the distal ileum and colon into the blood within minutes after eating [1, 2]. GLP-1 achieves its insulinotropic effects by binding to its specific receptor (GLP-1R), which positively stimulates insulin secretion, suppressing glucagon production, improving β-cell mass and function, inhibiting food intake, and slowing gastric emptying [3]. Hence, GLP-1/GLP-1R is a therapeutic target for treating type 2 diabetes mellitus (T2DM). Natural GLP-1 has a short half-life (1.5–2 min) because it is degraded by dipeptidyl peptidase-IV (DPP-4) [4]. Therefore, GLP-1 mimetics, such as exenatide, liraglutide, and taspoglutide, have been a focus of drug development in recent years [5–7]. However, all of the GLP-1 mimetics developed to date, or currently under development, are of peptidic nature, requiring multiple injections, and display some side-effects such as nausea and vomiting in the clinic [8]. Chen et al. recently reported 1,3-bis(4-((*p*-butoxycarbonyl)amino)benzamido)-2,4-bis(*m*-methoxy-*p*-(thiophene-2-methanoyl)phenyl)-cyclobutane-1,3-dicarboxylic acid (Boc5), with molecular weight of 1,076 Da, which is the first nonpeptidic GLP-1R agonist with potential pharmacological effects, including stimulating

R. Li
Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China

R. Li · H. Fan · Y. Gu · D. Si (✉)
State Key Laboratory of Drug Delivery Technology and Pharmacokinetics, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China
e-mail: ddysi@sohu.com

W. Liang
Institute of Biophysics Chinese Academy of Sciences, Beijing 100101, China

insulin secretion, elevating insulin sensitivity, reducing food intake, and slowing gastric emptying [9–11].

It is necessary to determine the in vivo pharmacokinetic profile of Boc5 for successful new drug development [12]. However, no analytical method for determination of Boc5 has been reported. Thus, we developed and validated a simple, robust, and sensitive high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) method to determine Boc5 in rat plasma.

Experimental

Chemicals and Reagents

Boc5 was obtained from the Institute of Biophysics Chinese Academy of Sciences (Beijing, China). Wogonin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol, acetonitrile, and formic acid were purchased from Tianjin Concord Tech Reagent Co. Ltd. (Tianjin, China). Analytical-grade ammonium formate was purchased from Tianjin Guang fu Fine Chemical Research Institute (Tianjin, China). Deionized water was prepared using a SYZ550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., Tianjin, China) and used throughout the study.

Instrumental and Analytical Conditions

Chromatographic analysis was performed using a Shimadzu HPLC system consisting of a binary LC-20AD delivery pumps, a DGU-20A5 Shimadzu vacuum degasser, a SIL-20AC Shimadzu autosampler, and a CBM-20A system controller (Shimadzu Scientific Instruments, Columbia, MD, USA). Separation of Boc5 and IS was achieved on an Atlantis T3 analytical column (100 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol, acetonitrile, and 20 mM ammonium formate addition to 2 % formic acid (39:39:22, *v*/*v*/*v*) at flow rate of 0.6 mL/min for 7.0 min. The column and autosampler were maintained at 40 and 4 °C, respectively.

The analytes were detected by an API 4000 QTRAP mass spectrometer (MDS Sciex, Toronto, Canada) equipped with electrospray ionization (ESI) in positive mode. Quantitation was performed using multiple reaction monitoring (MRM) mode, and the transitions monitored were *m*/*z* 1,077.2 → 784.4 for Boc5 and *m*/*z* 285.1 → 270.4 for IS with dwell time of 200 ms per transition. The working parameters maintained were as follows: curtain gas, 10 psi; gas 1, 40 psi; gas 2, 40 psi; collision gas, 8 psi; ion spray voltage, 5,000 V; heater temperature, 600 °C. The declustering potential, collision energy, entrance potential, and collision cell exit potential were optimized at 130, 40, 8, and 22 V, respectively. All data were acquired and processed using Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA).

Preparation of Standard and Quality Control

Stock solutions were prepared by dissolving Boc5 and wogonin in methanol to concentration of 1.0 mg/mL, respectively. Working solutions of Boc5 ranging from 0.02 to 20 μg/mL were prepared by diluting stock solution with methanol. IS working solution (2.5 μg/mL) was prepared by diluting IS stock solution with methanol. All working solutions were stored at −4 °C before use.

Calibration standards of Boc5 were prepared at concentrations of 2, 5, 10, 50, 200, 1,000, and 2,000 ng/mL by spiking 10 μL working solutions into 100 μL blank plasma. Quality control (QC) samples were prepared in the same manner at the three concentration levels of 5, 50, and 1,000 ng/mL.

Sample Preparation

To an aliquot of 100 μL plasma, 10 μL methanol and 10 μL IS working solution were added and vortex-mixed for 30 s. The mixture was precipitated with 300 μL methanol, vortex-mixed for 1 min, and then centrifuged at 12,000 rpm for 5 min. Supernatant (200 μL) was transferred into autosampler vials, and 5 μL was injected into the HPLC–MS/MS system for analysis.

Method Validation

Method validation and documentation were performed according to guidelines set by the US Food and Drug Administration (FDA) for bioanalytical method validation [13]. This method was validated in terms of specificity, linearity, lower limit of quantification (LLOQ), intra- and interday accuracy and precision, extraction recovery, matrix effect, and analyte stability during sample storage and processing procedures.

Specificity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma samples. Each blank sample was tested for interferences using the precipitation procedure and HPLC–MS/MS conditions described above. The plasma samples studied for specificity were normal rat plasma.

Calibration curves were obtained by least-squares linear regression of the peak area ratio of analyte to IS (*Y*-axis) versus the nominal analyte concentrations (*X*-axis) with a weighting factor of $1/x^2$. The LLOQ was defined as the lowest concentration on the calibration curve with

precision and accuracy within 20 % and signal-to-noise (S/N) ratio greater than 10.

Intraday precision and accuracy were determined by analysis of six replicates of each QC sample ($n = 6$) at low (5 ng/mL), medium (50 ng/mL), and high (1,000 ng/mL) concentration levels during a single analytical run. The same procedure was repeated on three consecutive days to determine interday precision and accuracy ($n = 18$). Precision was expressed as the relative standard deviation (RSD), and accuracy was described as the relative error (RE). Acceptable precision and accuracy should be within 15 %.

Sample dilution was validated with samples of concentration higher than 2,000 ng/mL, which were diluted 20-fold with blank rat plasma and processed as other QC samples.

Recovery was calculated by comparing the peak areas of Boc5 added into blank plasma and extracted using the protein precipitation procedure with those obtained from Boc5 spiked directly into post-protein precipitation solvents at three QC concentration levels ($n = 6$).

Endogenous substances may cause ion suppression or signal enhancement. Matrix effect was assessed by comparing the peak areas of Boc5 after addition of low, medium, and high concentrations of Boc5 to (A) mobile phase and (B) the supernatant of extracted blank plasma ($n = 6$). These studies were conducted with six different lots of rat plasma. The peak area ratio of B/A (as a percentage) or the percentage matrix factor was used as a quantitative measure of the matrix effect.

Stability of Boc5 in plasma samples was determined with respect to short-term, postpreparative, freeze–thaw, and long-term stability by using three QC sample sets in each case. Short-term stability was tested after storing the samples at room temperature for 6 h. Postpreparative stability was assessed by storing the prepared samples in the cooled autosampler (4 °C) for 12 h. Freeze–thaw stability was determined using QC samples after three freeze–thaw cycles. Long-term stability was tested by assaying the QC plasma samples after 30 days of storage at −20 °C. Stock Boc5 standard solution stability was tested after 2 months of storage under refrigeration conditions. The analyte was considered stable when the concentration difference was less than 15 %.

Application to Pharmacokinetic Study

Wistar rats (220 ± 20 g) were purchased from the Institute of Radiation Medicine, Chinese Academy of Medical Sciences and kept in a controlled (temperature: 25 ± 2 °C, humidity: 50 ± 20 %) room with natural light–dark cycle for 1 week before the experiment. The animal experiment was carried out according to the Guidelines for the Care and Use of Laboratory Animals of Tianjin Institute of Pharmaceutical Research, which was approved by Tianjin Municipal Science and Technology Commission (Tianjin, China). After Boc5 was intravenously administrated to rats at dose of 1 mg/kg [4 mg Boc5 dissolved in 0.5 mL dimethyl sulfoxide (DMSO) and 0.5 mL Tween-80, made up to 20 mL with 0.9 % NaCl], blood samples (0.3 mL) were collected in heparinized tubes via the orbital vein at 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h. After centrifugation, the plasma samples were collected, immediately stored at −20°C, and analyzed within 1 month. Pharmacokinetic parameters, including half-life ($t_{1/2}$), volume of distribution (Vd) and clearance (CL), were analyzed by noncompartmental methods using DAS 2.0 software (Chinese Mathematical Pharmacology Society).

Results and Discussion

HPLC–MS/MS Optimization

The ESI source was chosen for ion production, since Boc5 is a highly polar compound. To optimize mass-spectrometric conditions, a full scan was carried out in positive/negative ion detection mode. The results showed that the response of Boc5 in positive ion mode was much higher than that in negative mode. After collision-induced dissociation, the most abundant and stable product ions were at *m/z* 784.4 for Boc5 and at *m/z* 270.4 for IS (Fig. 1). Therefore, the MRM transitions of *m/z* 1,077.3 → 784.4 for Boc5 and *m/z* 285.1 → 270.4 for IS were selected for quantitative analysis.

Four types of column, namely Intersil C_{18} (4.6 mm × 150 mm, 5 μm), Kinetex C_{18} (4.6 mm × 100 mm, 5 μm), Symmetry C_{18} (4.6 mm × 100 mm, 5 μm), and Atlantis T3 (4.6 mm × 100 mm, 5 μm), were compared in terms of chromatographic parameters such as resolution, asymmetry, variability of retention time, and peak width. The Intersil C_{18} column was excluded because of its poor separation and delayed retention time. Since Boc5 is an acidic compound and is strongly polar, both Kinetex C_{18} and Symmetry C_{18} columns showed weak response and poor peak shape, providing more proof that both of these columns offer higher selectivity (response) for alkaline compounds [14, 15]. According to the manufacturer's instructions, the Atlantis T3 column could be the best choice for separation of Boc5, and indeed our experiment proved that the Atlantis T3 column provided suitable retention time, excellent chromatographic separation, symmetric peak shape, and appropriate resolution.

To choose the optimal mobile phase, various mixtures of solvents such as methanol, acetonitrile, and methanol/acetonitrile (50:50, *v/v*), with typical buffers such as formic

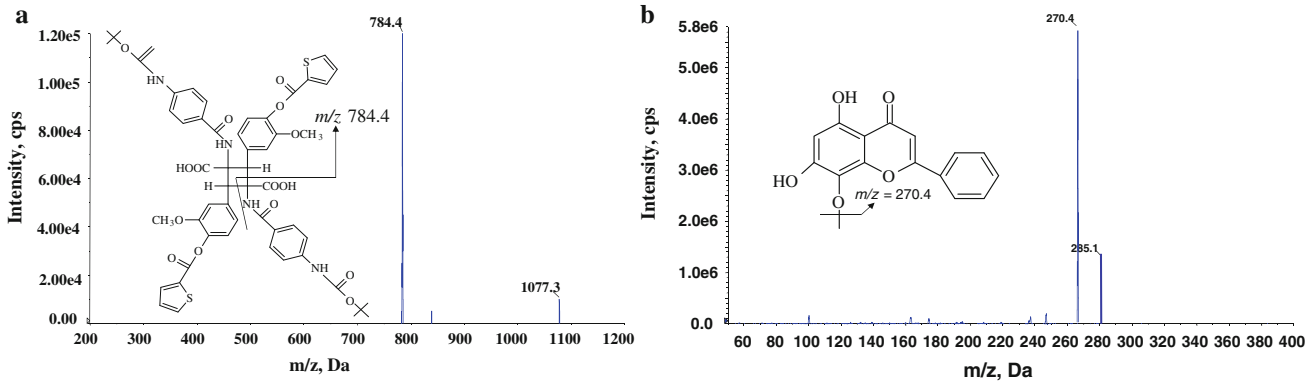

Fig. 1 Full-scan product ion spectra of $[M + H]^+$ form Boc5 (**a**) and wogonin (**b**)

acid and ammonium formate, were also tested. Boc5 standard solutions diluted with methanol, acetonitrile, and methanol/acetonitrile (50:50, *v*/*v*) were directly infused into the mass spectrometer to evaluate for scanning, respectively. The MS signal intensity of Boc5 in methanol/acetonitrile solution (50:50, *v*/*v*) was much higher than that in methanol or acetonitrile solutions. Meanwhile, we found that the peak shapes of Boc5 and IS were dramatically improved when the mobile phase was added to 20 mM ammonium formate, and the MS ionization efficiency of Boc5 was obviously enhanced when the mobile phase was added to 2 % formic acid. Consequently, a mixture of methanol, acetonitrile, and 20 mM ammonium formic containing 2 % formic acid (39:39:22, *v*/*v*/*v*) was adopted in this study, providing symmetric peak shapes of Boc5 and IS as well as high sensitivity.

According to FDA guidance, an IS in the analysis of biological samples could be a structurally similar analog of the analyte or a stable labeled compound. A deuterated standard would be preferred as the IS in a HPLC–MS assay. However, considering radiation exposure and the high cost of the deuterated IS, deuterated Boc5 was not chosen as the IS in the study. Due to the large structure of Boc5, synthesis of analogs is difficult. In fact, a compound with similar structure, extraction recovery, and chromatographic and mass-spectrometric behavior to the analyte may also be considered. In the study, wogonin was selected as the IS based on its similar chromatographic retention and mass-spectrometric behavior to Boc5, and the results of method validation demonstrated that the extraction recovery and matrix effect of wogonin were similar to those of Boc5, being stable in the sample process.

In this work, the plasma samples were processed by protein precipitation with methanol, and the extracted supernatant samples were injected directly for HPLC–MS/MS analysis. Protein precipitation has the advantages of a simple sample preparation procedure and excellent reproducibility.

Method Validation

The analytical method was shown to be selective for Boc5 and IS, as concluded from the absence of interferences in different blank plasma samples at the retention time of either Boc5 or IS. Typical chromatograms of blank plasma, plasma sample spiked with Boc5 and IS, and plasma sample from a rat after administration are shown in Fig. 2. The retention times of Boc5 and IS were 5.6 and 3.6 min, respectively.

Nine calibration curves were performed during the method validation. Good linearity was shown over the concentration range of 2–2,000 ng/mL for Boc5 in rat plasma. The typical calibration curve of Boc5 in rat plasma was $Y = 0.00069X + 0.00073$ ($r = 0.9960$). The LLOQ was found to be 2 ng/mL with accuracy of 10.0 % and precision of −3.0 %.

The intra- and interday precisions and accuracies of QC samples are summarized in Table 1. The intra- and interday precisions ranged from 2.6 to 5.1 % and 5.7 to 7.3 % at three QC levels, respectively. The relative error values were 3.0, −3.6, and −2.1 % for intraday accuracy and 1.6, −3.2, and −0.7 % for interday accuracy at three concentration levels of 5, 50, and 1,000 ng/mL, respectively. These values are well within the acceptable criteria of precision less than 15 % and accuracy within ±15 %.

To assess the accuracy and precision of dilution, six replicated samples of dilution were prepared at concentration of 40 μg/mL, and diluted 20-fold. The precision was 2.7 %, and the accuracy was 7.2 %, indicating that these samples after diluting with blank plasma could be accurately determined.

Summaries of matrix effect and recovery data are presented in Table 2. Plasma samples could be reliably processed with protein precipitation, as recovery ranged from 93.4 to 100.2 % across the entire validation range. The matrix effects of Boc5 were 93.8 ± 17.4, 88.0 ± 1.1, and 88.7 ± 3.5 % at three concentrations of 5, 50, and

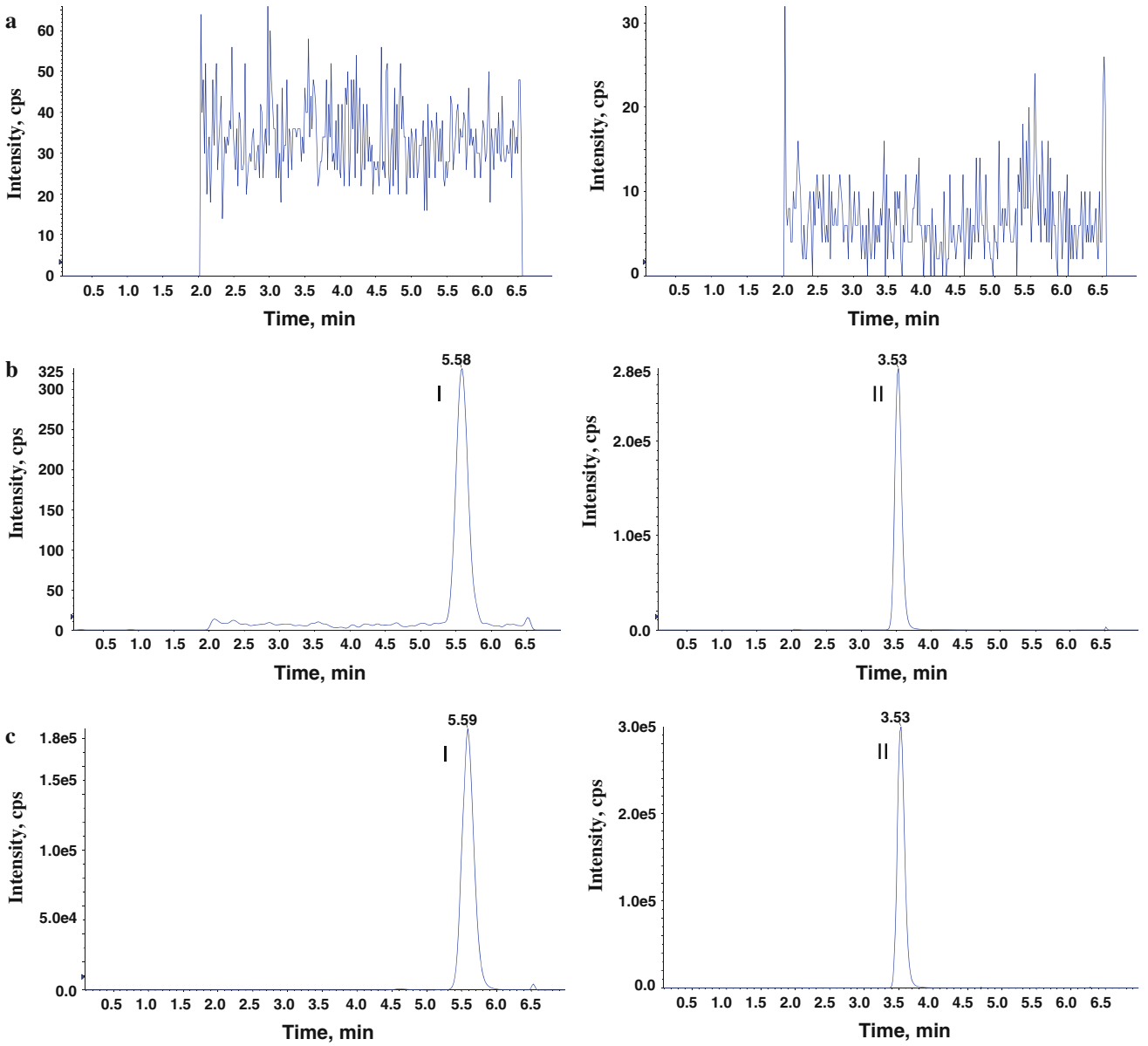

Fig. 2 Typical MRM chromatograms of Boc5 (I) and wogonin (II) in rat plasma samples: **a** blank rat plasma, **b** blank rat plasma spiked with Boc5 (2 ng/ml) and IS (2.5 μg/mL), and **c** rat plasma at 3 h after intravenous administration of Boc5 to rat

Table 1 Precision and accuracy of the assay method for Boc5 in rat plasma

Concentration	Mean ± SD	Precision (%)	Accuracy (%)
Intraassay ($n = 6$)			
LLOQ (2.0 ng/mL)	1.9 ± 0.2	10.0	−3.0
QC1 (5.0 ng/mL)	5.2 ± 0.03	5.1	3.0
QC2 (50 ng/mL)	48.2 ± 2.3	4.8	−3.6
QC3 (1,000 ng/mL)	979.0 ± 43.9	2.6	−2.1
Interassay ($n = 3$)			
QC1 (5.0 ng/mL)	5.1 ± 0.4	5.7	1.6
QC2 (50 ng/mL)	48.4 ± 2.3	7.3	−3.2
QC3(1,000 ng/mL)	993.0 ± 43.9	6.0	−0.7

Table 2 Matrix effects, recovery, and stability of Boc5 (IS) in rat plasma

Validation parameter	*n*	Q1 (5 ng/ml)	Q2 (50 ng/ml)	Q3 (1,000 ng/ml)	IS (2.5 μg/mL)
Matrix effect (%)	6	93.8 ± 17.4	88.0 ± 1.1	88.7 ± 3.5	100.6 ± 0.6
Recovery (%)	6	93.4 ± 10.8	93.4 ± 2.5	102.0 ± 3.7	102.0 ± 0.5
Short-term stability (%)	6	105.3 ± 2.3	99.2 ± 1.6	100.8 ± 1.9	
Autosampler stability (%)	6	93.7 ± 3.1	112.7 ± 4.9	114.3 ± 2.1	
Freeze–thaw stability (%)					
3rd cycle	6	103.9 ± 7.0	97.8 ± 1.4	103.5 ± 4.4	
Long-term stability (%)	6	94.0 ± 2.3	99.1 ± 8.8	94.9 ± 3.1	
Stock solution	6	105.3 ± 7.8	88.0 ± 1.9	88.5 ± 4.2	

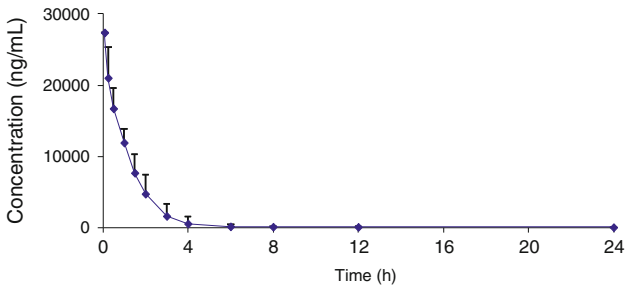

Fig. 3 Mean plasma concentration–time profile of Boc5 after a single intravenous administration dose of 1 mg/kg of Boc5 to rats ($n = 6$)

1,000 ng/mL, respectively, while the matrix effect of IS was 100.6 ± 0.58 %. The deviation for medium QC samples spiked in six lots of plasma was less than 10 % (data not shown), indicating that the relative matrix effect for Boc5 in rat plasma was negligible. This result is in agreement with international guidelines and indicates low ion suppression [13, 16]; considerable retention of the analyte and IS most likely contributed to this desirable lack of ion suppression.

Stability was investigated under different experimental conditions to which samples might be exposed. The results were obtained by comparing the freshly prepared samples with those handled as described above. The results are presented in Table 2. All values were within ±15 % of nominal, indicating good stability of analytes in rat plasma.

Application to Pharmacokinetic Study

The validated method was applied to study the pharmacokinetics of Boc5 after intravenous administration to rats at dose of 1 mg/kg. The mean plasma concentration–time curve of Boc5 is presented in Fig. 3. A noncompartmental pharmacokinetic model was used to calculate the pharmacokinetic parameters of Boc5 following intravenous administration. Boc5 was quickly eliminated with systemic clearance of 31.9 ± 5.0 L/h/kg. The elimination half-life was 4.6 ± 0.3 h. The volume of distribution was 213.1 ± 43.3 L/kg, suggesting that Boc5 is extensively distributed in rat.

Conclusions

A simple, robust, and sensitive HPLC–MS/MS method for quantitation of Boc5 in rat plasma samples was developed and validated for the first time. The method uses a simple protein precipitation which can simplify the sample preparation procedure and provide excellent reproducibility, and the runtime of 7 min enables high sample throughput. This HPLC–MS/MS method has been successfully applied to pharmacokinetic evaluation of Boc5 after intravenous administration to rats, and is currently being applied for further pharmacokinetic characterization of Boc5.

Acknowledgments This research was supported by Major State Basic Research Development Program of China ("973" Program) (no. 2010CB735602 and no. 2010CB933904).

References

1. Kieffer TJ, Habener JF (1999) Endocr Rev 20:876
2. Kim W, Egan JM (2008) Pharmacol Rev 60:470–512
3. Neumiller JJ (2009) Am Pharm Assoc 49:16
4. Meier JJ, Nauck MA, Kranz D, Holst JJ, Deacon CF, Gaeckler D, Schmidt WE, Gallwitz B (2004) Diabetes 53:654
5. Van Genugten RE, Van Raalte DH, Diamant M (2009) Diabetes Res Clin Pract 86:26–34
6. Croom KF, McCormack PL (2009) Drugs 69:1985
7. Lotfy M, Singh J, Kalász H, Tekes K, Adeghate E (2011) Open Med Chem J 5:82–92
8. Kieffer T, McIntosh CH, Pederson RA (1995) Endocrinology 136:85
9. Chen DS, Liao JY, Li N, Zhou CH, Liu Q, Wang GX, Zhang R, Zhang S, Lin LL, Chen KX, Xie X, Nan FJ, Young AA, Wang MW (2007) Proc Natl Acad Sci USA 104:943
10. Su HR, He M, Li HM, Liu Q, Wang J, Wang YQ, Gao WW, Zhou L, Liao JY, Young AA, Wang MW (2008) PLoS ONE 3:2892
11. Wang MW, Liu Q, Zhou CH (2010) Acta Pharmacol Sin 1026:31
12. Murphy KG, Bloom SR (2007) Proc Natl Acad Sci USA 104:689
13. FDA. Guidance for Industry, Bioanalytical Method Validation, FDA. http://www.fda.gov/cder/guidance

14. Grobosch T, Schwarze B, Stoecklein D, Binscheck T (2012) J Anal Toxicol 36(1):36–43
15. Uniyal GC, Bala S, Mathur AK, Kulkarni RN (2001) Phytochem Anal 12(3):206–210
16. Koufopantelis P, Georgakakou S, Kazanis M, Giaginis C, Margeli A, Papargiri S, Panderi I (2009) J Chromatogr B 877(30):3850–3856