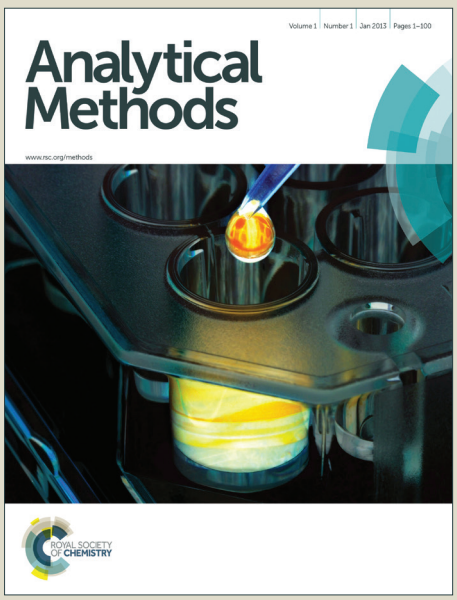
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Quantification of Recombinant Human Relaxin-2 (B-29/A-24) in non-pregnant rat plasma using ultra performance liquid chromatography–mass spectrometry

Xiaoyan Zhu[a,b], Jingjing Liu[a], Jie Wu[a] , Rongyue Cao[a] and Taiming Li [*a],

Abstract

A rapid, sensitive and high throughput method using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was first developed for the determination of Recombinant Human Relaxin-2 (B-29/A-24) in non-pregnant rat plasma for characterizing the pharmacokinetics. The method was operated under pseudo-multiple reaction monitoring in the positive electrospray ionization mode. H2 Relaxin and internal standard (Levemir) were extracted under acidic conditions by one-step protein precipitation with acetonitrile. Chromatographical separation was obtained on a XBridge BEH300 C4 column with a gradient elution profile consisting of acetonitrile and 0.2% aqueous formic acid. The method was fully validated in terms of linearity, selectivity, precision, accuracy, recovery, matrix effect and stability. The assay was validated over a concentration range of 10.0 –1000 ng/mL and no interfering peaks were detected at the retention time of H2 Relaxin and internal standard in blank rat plasma. Recoveries from spiked controls were >83% for the analytes at all QC levels and no obvious matrix effects were found. Stability studies indicated that H2 Relaxin in rat plasma underwent no significant degradation. In conclusion, this method was successfully applied to determine the concentration of H2 Relaxin in plasma collected from Sprague–Dawley rats during the pharmacokinetic study of H2 Relaxin.

Keywords: Recombinant Human Relaxin-2; pseudo-MRM mode; UPLC–MS/MS; rat plasma

1. Introduction

Relaxin, which was first investigated in 1926 by Frederick Hisaw, had been well-known as a reproductive peptide hormone involved in pregnancy, parturition and lactation.[1-2] This peptide hormone family is comprised of seven members: the relaxin genes RLN 1, RLN 2 and RLN 3 and the insulin-like peptide genes INSL 3, INSL 4, INSL 5 and INSL 6.[2] Among these, the human RLN2 gene, also called Recombinant Human Relaxin-2 or H2 Relaxin is the major stored and circulating isoform of relaxin in human blood and will be the form of relaxin discussed in this paper.[3] Like Insulin, H2 Relaxin (B-29/A-24) is a 6 kDa, 53 amino acid nonglycosylated, heterodimeric polypeptide which is made up of two disulfide-linked chains, A and B.[4] To date, scientists have noticed that H2 Relaxin has diverse physiological and pathological effects, with significant therapeutic and clinical implications, highlighting its importance in humans.[4-5] It plays a vital role in the hemodynamic and renovascular adjustment[6] which lead to evaluation in Phase II/III clinical trials for the treatment of congestive heart failure and acute heart failure in 2006.[3,5,7-8] Nair et al. have reported that relaxin can be highly expressed by cancer cells, acting on its receptor to promote cancer growth and metastasis.[7] These various findings have shown light on the relationship between H2 Relaxin and cancers. In this paper, H2 Relaxin (B-29/A-24) is derived from *E.coli* cells, with the ED_{50} at 5.6 - 6.7 ng/mL. The ED_{50} is determined by its ability to induce cAMP accumulation in THP-1 human acute monocytic leukemia cells.[9]

It was reported that immunoassay method has been used in the quantification of this peptide. Kraynov et al. had reported the use of enzyme-linked immune-sorbent assay (ELISA), radioimmunoassay (RIA), electrochemiluminescence immunoassay (ECLIA) and bridging electrochemiluminescence assay (bridging ECLA) to determine the PEG-Relaxin and wild type recombinant human Relaxin (wt rhRelaxin).[10] Paccamonti et al. determined plasma concentrations of relaxin in cows with various delivery vehicles and routes of administration by RIA.[11] High sensitivity (~10 pg/mL for H2 Relaxin) and high sample throughput are the vital virtues of immunoassay. However, the risk of cross-reactivity and the limited linear dynamic ranges, leading to the multiple dilutions of samples, can also compromise the accuracy of quantification.[12-14]

The determination of peptide and protein by UPLC-MS/MS can offer improved accuracy and precision and low cost was another factor that should not be neglected. Relaxin is a 6 kDa peptide, which is suitable for determination by mass, and need only multiply charges to achieve an ion within the mass range of the instruments. The extraction techniques of peptide and protein are the same with small molecular compounds, including solid phase extraction (SPE), protein precipitation (PP) and liquid–

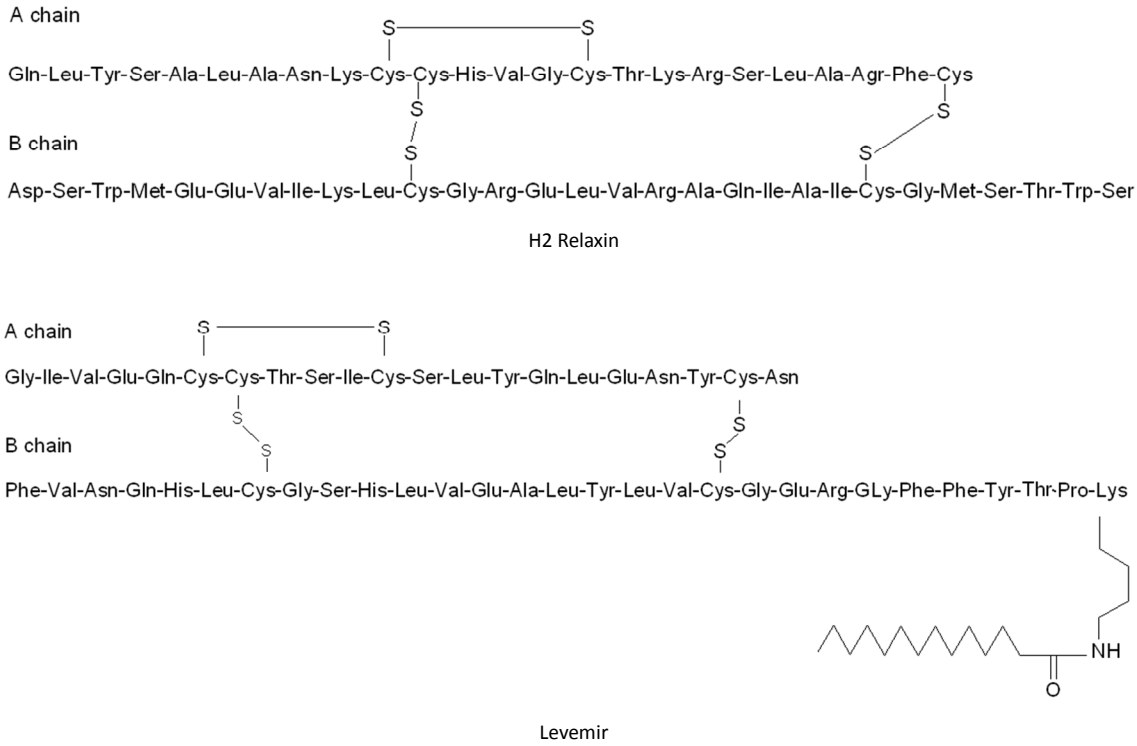


Fig. 1 Amino acid sequences and disulfide bridges of H2 Relaxin and Levemir (IS)

liquid extraction (LLE). The usefulness of LLE is limited for large molecules because most peptides are usually aqueous. If there is no endogenous interference, PP is a quick and simple technique by adding a precipitating solvent or acid which can provide good results at low cost. When PP gives poor results, SPE is another choice which can provide better sensitivity, selectivity, low matrix effect and high recovery.[13-14] The poor fragmentation leading to low sensitivity is another challenge of bioanalysis because peptide bonds are nearly equal in energy to each other.[14-15] Pseudo-MRM is an effective way to overcome this with the same Q1 and Q3 ions and low collision energy. At the same time, we should make sure that there are no interferences at the retention time of the target peptide or protein.

The main aim of the present work was to introduce a simple, sensitive and accurate UPLC-MS/MS quantitative method using PP and pseudo-MRM for generating the pharmacokinetic profile of H2 Relaxin in non-pregnant Sprague-Dawley rat plasma for the first time.

2. Experimental

2.1. Reagents and standards

H2 Relaxin (B-29/A-24, MW=5963) was provided by Shanghai Hengrui Pharmaceutical Co., Ltd. (Shanghai, China) and the purity was above 95%. Levemir (internal standard, MW=5917) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark) and each milliliter of solution contains 100U (equivalent to 14.2 mg) insulin determir. Their amino acid sequences are shown in Fig. 1. HPLC grade acetonitrile was obtained from Fisher Scientific (Waltham, MA, USA). DMSO and Formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Purified water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). HCl and other chemicals were of analytical grade. 96-well conical btm PP microplates (0.45 mL well) were obtained from Thermo Scientific (Waltham, MA, USA). Low-protein bind polypropylene tubes were purchased from Corning (Axygen®, Tewksbury, MA, USA).

2.2. Instrumentations and operating conditions

The UPLC–MS/MS system consisted of an API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystems /MDS-Sciex, Foster City, CA, USA) coupled to a Shimadzu Nexera UHPLC LC-30A (Shimadzu Corporation, Kyoto, Japan). Mass calibration, data acquisition and quantitation were controlled by the Analyst 1.6 Software. Chromatographic separation of H2 relaxin was performed on a C4 analytical column (XBridge™ BEH300, 100 mm×4.6 mm, 3.5 μm; Waters, Milford, MA, USA) with a gradient formed between 0.2% aqueous formic acid (A) and acetonitrile (B), at a flow rate of 0.8 mL/min. The gradient cycle consisted of an initial 1.0 min isocratic segment (80% A and 20% B) increasing solvent B to 60% within 5 min and maintained from 6.0 to 6.5 min. Then changing back to 20% solvent B at 7.0 min and maintaining up to 10.0 min for column equilibration. The temperature of autosampler tray was set at 4 ˚C.

The mass spectrometer was tuned to the following operating parameters for optimal performances: source temperature 500 ˚C,

ion spray voltge (IS) 5000 V, curtain gas (CUR) 20 psi, collision gas (CAD) 10 psi, entrance potential (EP) 10 V, collision cell exit potential (CXP) 25 V, ion source gas 1 (GS_1) and ion source gas 2 (GS_2) 50 psi. Quantification was thus performed using pseudo-multiple reaction monitoring (pseudo-MRM) of m/z 994.9→994.9 for H2 relaxin and multiple reaction monitoring (MRM) of m/z 1184.7→454.5 for IS, respectively. Declustering potential (DP) and collision energy (CE) were set at 120 V and 6 eV for H2 relaxin and 100 V and 36 eV for Levemir, respectively. A dwell time of 150 ms was used for each ion transition. Unit resolution was set for both Q1 and Q3 mass detection. The scan results are shown in Fig. 2 and Fig. 3.

2.3. Preparation of stock Solutions, calibration curves and quality control samples

The stock solution at the concentration of 1.13 mg/mL was prepared in 0.05 mM HCl for H2 Relaxin. The solution was further serially diluted with DMSO to obtain standard working solutions over a concentration range of 100-10000 ng/mL. The stock solution of Levemir was diluted with DMSO/ACN (50:50, V/V) to make a 2.5 μg/mL spiking solution. The working solution of Quality control (QC) samples at three concentrations (200, 2000 and 8000 ng/mL) for H2 Relaxin were used both in pre-study validation and during the PK study.

2.4. Extraction procedure

QC samples, calibration standards and rat plasma samples were extracted employing a protein precipitation technique. 45 μL of blank rat plasma was spiked with 5 μL of working solution to achieve concentrations of 10.0, 20.0, 50.0, 100, 200, 500 and 1000 ng/mL for calibration standards and 20.0, 200 and 800 ng/mL for QC samples. For rat plasma samples, each tube contained 50 μL of plasma. After adding 10 μL of 2.5 μg/mL of IS and 10 μL of 5% HCl, samples were precipitated with 150 μL acetonitrile, and the mixture was then vortexed for 3 min. The samples were then centrifuged for 10 min at 15,000 rpm twice at 4 °C. Each supernatant was transferred to 96-Well Microplate and the injection volume was set at 30 μL. Calibration curves were constructed by weighted linear regression ($1/x^2$) which was determined to be the best fit due to the wide concentration range investigated.

2.5. Method validation

The method was fully validated for its linearity, selectivity, precision, accuracy, recovery, matrix effect, Low limit of detection (LOD), Lower limit of quantification (LLOQ) and stability. LLOQ was defined as the lowest concentration on the calibration curve with a precision of 20% and accuracy of 80-120%. The intra-batch precision and accuracy were measured by analyzing five spiked samples of H2 Relaxin at each QC level. The inter-batch precision and accuracy were determined over three days by analyzing QC samples per day.

Recovery was calculated using the ratio of peak area with analyte and IS compared by QC samples and samples spiked with H2 Relaxin after blank plasma samples were processed. Matrix effect on the ionization efficiency was evaluated using the ratio of peak area with analyte and IS compared in spiked plasma extract and in solvent system, respectively.

Stability of H2 Relaxin in rat plasma was investigated by analyzing five replicate QC samples at three concentration levels. QC samples were subjected to one freeze-thaw cycle at -80 ºC for a week and were stored at ambient temperature for 4 h before sample processing and analyzing. The post-preparative stability of H2 Relaxin was also investigated in the autosampler at 4 ºC for 8 h. The stability of the working solutions of H2 Relaxin and IS was evaluated by testing their validity over three months when stored refrigerated.

2.6. Application to pharmacokinetic study

Six male and six female Sprague–Dawley rats (160 ~ 180 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China, certificate No. SCXK-2008-0016) and housed with free access to food and water. All animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University and were in accordance with the Guidelines. After a 12 h fast, the rats were administered H2 relaxin at 0.5 mg/kg by subcutaneous and intravenous injection in physiological saline.

About 0.2 mL of blood samples via the postocular vein were collected into low-protein bind polypropylene tubes before dosing and at 5, 15 and 30 min, 1, 1.5, 2, 3, 4 h after dosing for s.c., 2, 5, 15 and 30 min and 1, 1.5, 2, 4 h for i.v.. Ethylene Diamine Tetraacetic Acid (EDTA), a metalloprotease inhibitor, was used as an anticoagulant to inhibit potential metal-catalyzed oxidation. Blood samples were centrifuged immediately at 4 °C to obtain plasma and were stored at −80 °C until analysis. All samples were split into two aliquots before freezing, and each one has 50 μL of rat plasma samples.

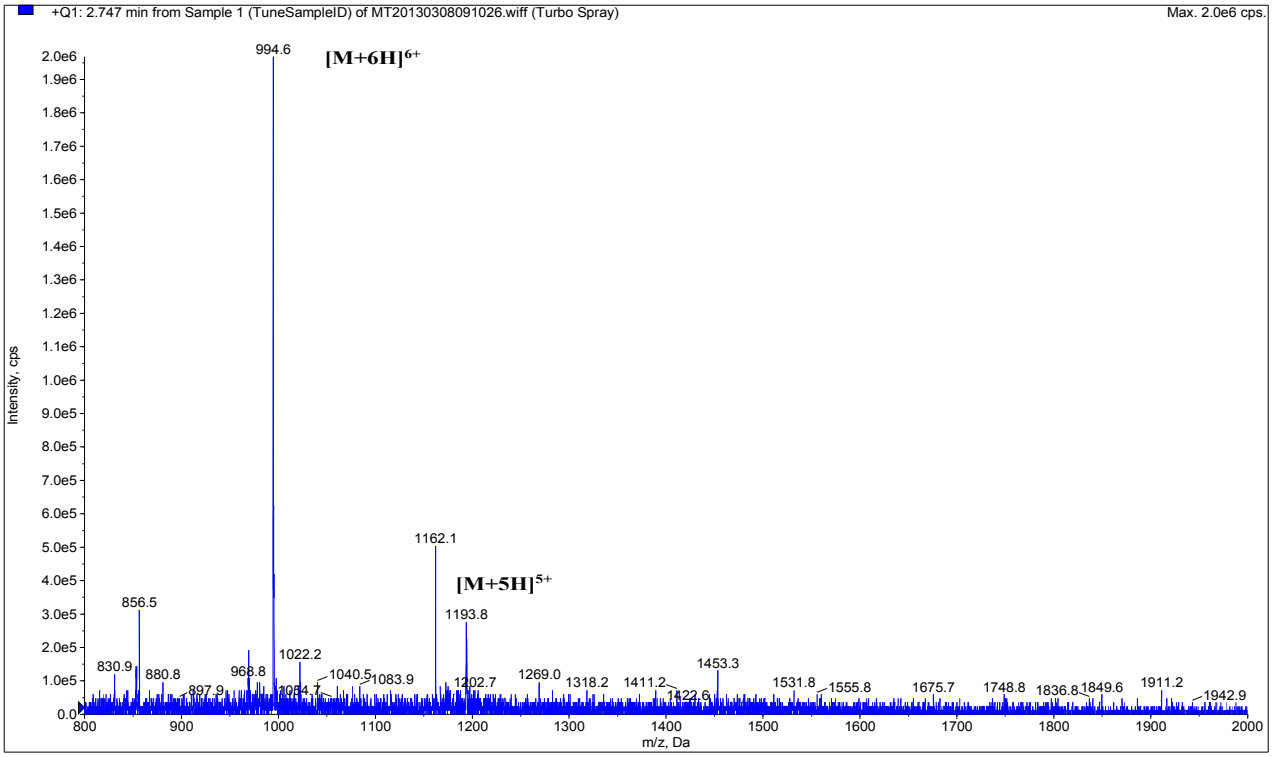
Pharmacokinetic parameters were calculated with noncompartmental analysis by Phoenix™ WinNonlin (Pharsight, version 6.1). Bioavailability (F%) was calculated from the ratio of $AUC_{0-\infty}$ with subcutaneous and intravenous injection.

3. Results and discussions

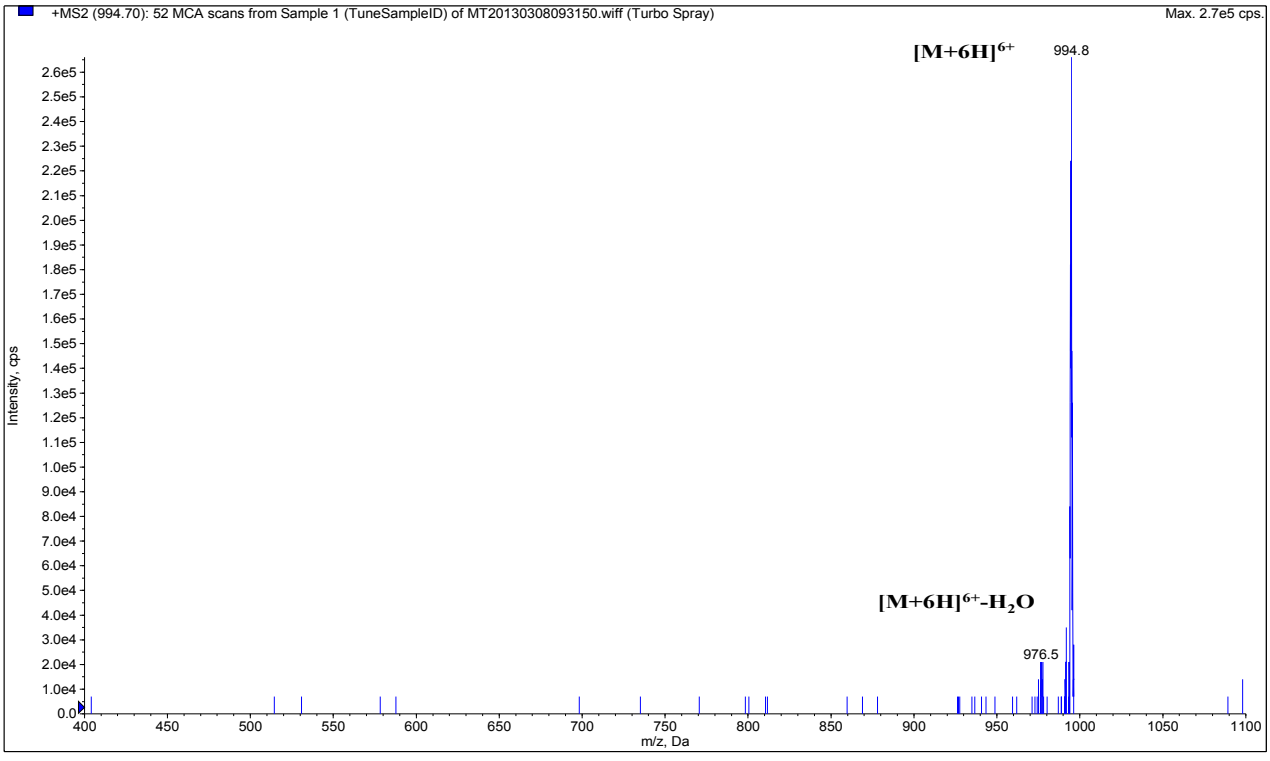
3.1. Method optimization

The choice of positive or negative ion mode was evaluated at the early stage of method development. The positive ion mode was selected since it produced higher intensity signals than the negative ion mode. The full scan mass spectrum and the product ion spectrum for Relaxin and IS are shown in Fig. 2 and Fig. 3. $[M+6H]^{6+}$ ion for Relaxin at m/z 994.9 and $[M+5H]^{5+}$ ion for IS at m/z 1184.7, being the more abundant of these molecular ion signals, were chosen as precursor ions. One product ion of H2 Relaxin in the spectrum was m/z 976.5 ($[M+6H]^{6+}$-H_2O), which was the less abundant fragment with the low ion signals. Pseudo-MRM (m/z 994.9→994.9) may be the only choice for the poor fragement of H2 Relaxin. Fortunately, no significant interference was observed at the retention time of 3.70 min for H2 Relaxin. Two product ions of IS in the spectrum were m/z 454.5 (B (y_2))[17] and 1180.5 ($[M+5H-H_2O]^{5+}$). Finally, the product ion at m/z 454.5 was chosen as a sensitive product ion because of the abundant signal and high selectivity.

A number of commercially available columns, including



(I)



(II)

Fig. 2 Full scan mass spectrum of H2 Relaxin (I) and product ion spectrum of the mass selected $[M+6H]^{6+}$ ion of m/z 994.8 (II)

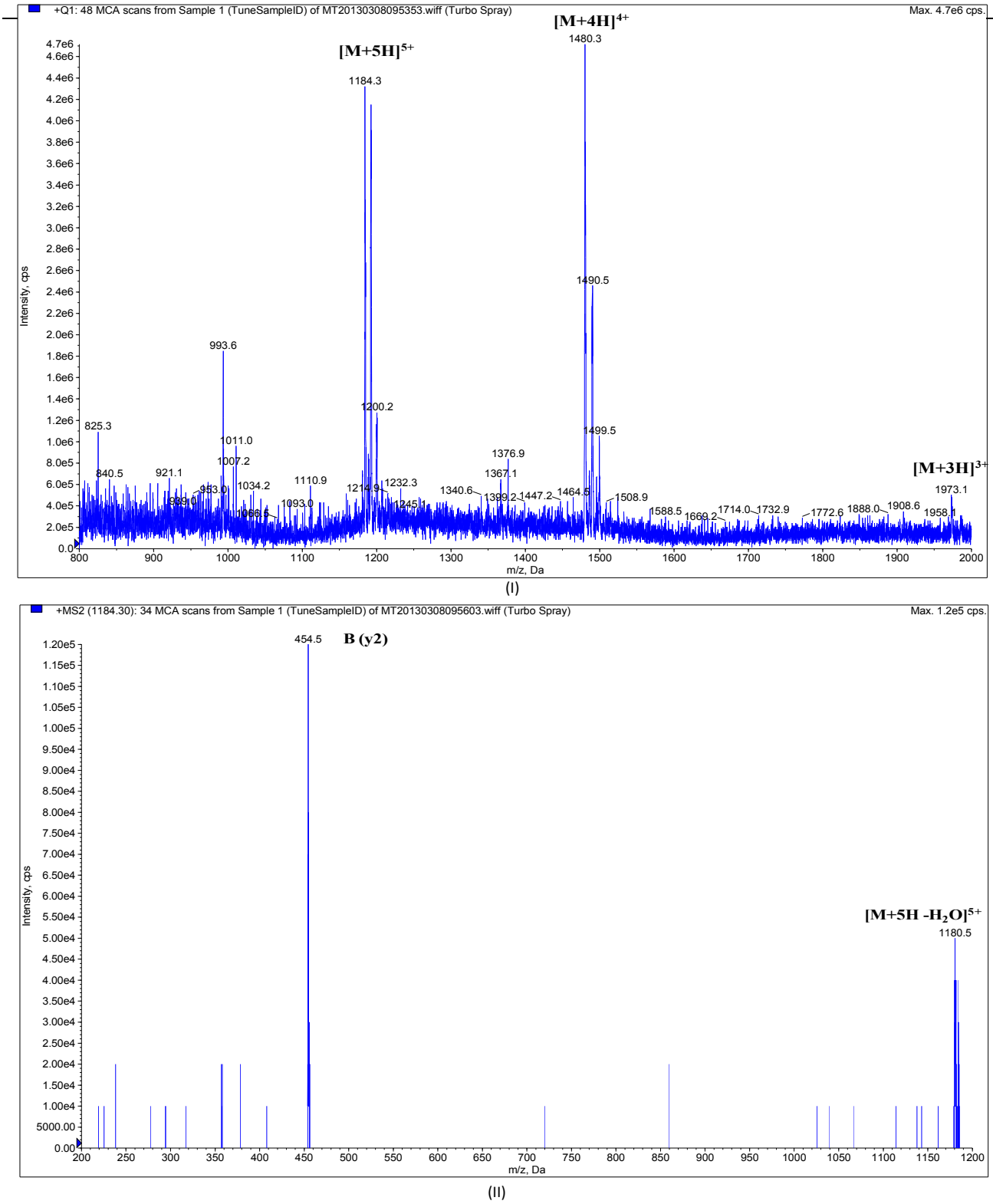


Fig. 3 Full scan mass spectrum of IS (I) and product ion spectrum of the mass selected $[M+5H]^{5+}$ ion of m/z 1184.7 (II).

Waters HPLC C4 column (BEH300, 100 mm×4.6 mm, 3.5 µm), Waters HPLC C18 column (BEH300, 100 mm×4.6 mm, 3.5 µm) and Waters UPLC C18 column (BEH130, 100 mm×2.1 mm, 1.7 µm) were tested and Waters HPLC C4 column provided a sharper peak shape. Waters HPLC C18 column provided a low peak height and ugly peak shape, yet the column joint of Waters UPLC column could not fit with Shimadzu UPLC which needed an interface. As for the mobile phase, 0.2% formic acid was found to be an important factor for acquiring the high sensitivity and the improved peak shape.

A stable isotopically Labeled (SIL) internal standard is the best choice for bioanalysis of peptides; however, the synthesis of SIL compounds is more difficult and expensive.[13-14] Insulin, the structural analogs of H2 Relaxin, is a useful and cheaper

alternative. Relaxin and insulin have the similar MW and structures, consisting of two chains, which are covalently linked by two inter-disulfides with an intra-disulfide link in the A chain. Human insulin (MW=5807) was once tested as IS due to its similar structure, however the poor fragment ($[M+5H-H_2O]^{5+}$, m/z 1162.4 → 1158.5)[16] and the similar retention time may interfere with H2 Relaxin. Levemir was adopted as IS finally because of the suitable retention time and the minimal endogenous interferences in the MRM channel (m/z 1184.7→ 454.4).[17]

As discussed, the greatest adsorption of protein and peptide is a challenge of analysis. The adsorption of H2 relaxin was tested by 5 consecutive transfers of QC solution from one vessel to another at room temperature every 15 min at the beginning of the study.[14] After 5 consecutive transfers, the response of H2 Relaxin and IS was reduced by 16.40% and 6.97%. Then, low-protein bind polypropylene tubes and DMSO were chosen for avoiding the adsorption of Relaxin. DMSO was used because it can promote the molecules to stay in solution rather than to adsorb to the vessel walls.[14] Moreover, the structure of proteins was easily modified by proteases in rat plasma.[14] Addition of hydrochloric acid was another key factor for the method which was a simple way to inhibit protease and improve response. However, large amount of DMSO and HCl do harm to the column and instrument. The different levels of HCl were tested and adding 10 μl of 5% HCl (0.227%, v/v) was the best choice.

3.2. Method Validation

3.2.1. Selectivity. Fig. 4 shows the representative chromatograms of blank rat plasma (a) and plasma sample obtained at 1 h after subcutaneous administration (b), supporting the high selectivity of this method. Relaxin and IS were baseline separated chromatographically with the retention time of above 3.70 and 5.02 min, respectively. According to the chromatograms, several interfering peaks caused by pseudo-MRM were detected between 4.6 and 5.5 min, which were not interfere with the target peptide.

3.2.2. Linearity of Calibration Curves. In the method, good linear response was observed in the concentration range 10.00–1000 ng/mL for H2 Relaxin. The calibration plots were constructed by weighted ($1/x^2$) least-squares regression. A typical regression equation for the concentration versus the peak area was y = 0.0114 x-0.123 (r=0.9977) and LLOQ was 10.00 ng/ml for H2 Relaxin with an accuracy of 112.85% and a precision of 7.60%. The LOD, defined as a signal–noise ratio over 3, was 5 ng/mL for Relaxin in plasma.

3.2.3. Precision and Accuracy. Intra- and inter-day precisions and accuracies of H2 Relaxin are shown in Table 1. In this assay, the intra- and inter-day precisions were within 10.35% and the accuracies ranged from 86.59 to 98.40% for H2 Relaxin, indicating the method is robust for the determination of H2 Relaxin.

3.2.4. Recovery and Matrix Effect

Data of extraction recoveries and matrix effects are collected in Table 2. The mean absolute recoveries were more than 83.20% for all the analytes (n = 5) indicating the efficiency of the sample preparation with little variation.

The matrix effects of Relaxin were within 89.62–97.53% which indicated that no significant endogenous matrix effects at three different concentration levels.

3.2.5 Stability. The stock solutions of H2 Relaxin and IS were proved to be stable for at least 3 months at 4 °C. H2 Relaxin was stable in rat plasma for 4 h at room temperature (20~25 °C) and the processed sample was stable in the autosampler for 8 h. No significant degradation in plasma samples after storage at −80 °C for one week.

3.3. Pharmacokinetics of Relaxin in rat plasma

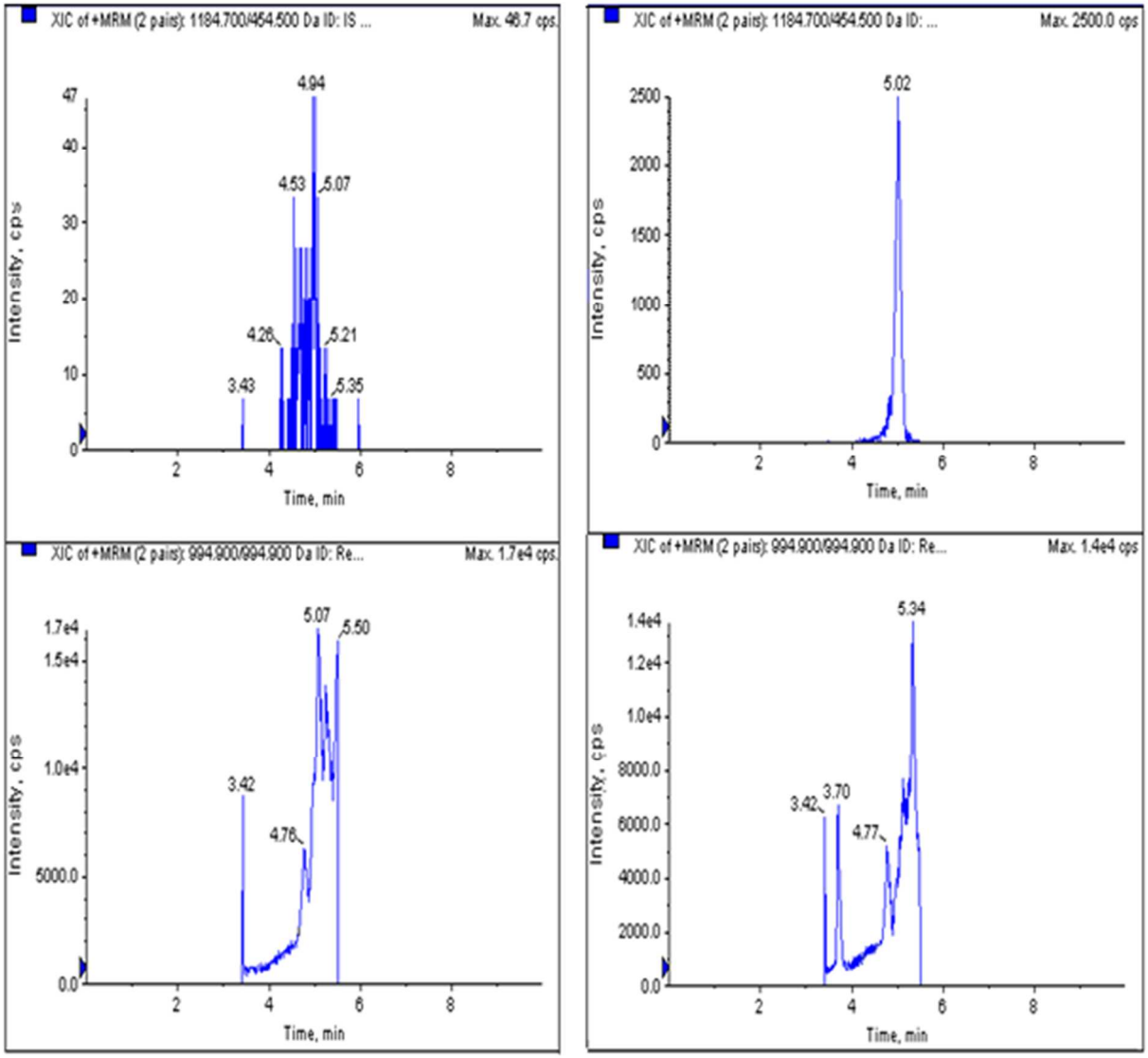
The plasma concentration–time profiles of H2 Relaxin in rats after a single s.c. and i.v. dose of 0.5 mg/kg are shown in Fig. 5. After s.c. administration, the peak plasma concentration was reached in rats at about 0.67 h, which indicated its rapid absorption. The half life was 1.23±0.32 h for s.c. and 0.97±0.48 h for i.v., revealing that the residence time for H2 relaxin in rats was very short. The systemic exposure of H2 Relaxin appeared to be comparable after subcutaneous and intravenous injection in rats with the same dosage, which was confirmed by the high subcutaneous bioavailability, with a mean value of 95.20%. All these parameters indicated that H2 Relaxin had a better PK characteristic in rats, and these pharmacokinetic parameters are similar to those reported by Kraynov et al. and the detailed data are shown in Table 3.

Table 2 Recovery and matrix effect of H2 relaxin from rat plasma (n=5, Mean±S.D.)

Spiked Concentration (ng/mL)	Recovery (Mean ± S.D., %)	Matrix effect (Mean ± S.D., %)
20.00	100.67±10.89	97.53±10.29
200.00	101.86±5.64	91.50±6.64
800.00	83.20±5.55	89.62±8.59

Table 1 The inter-batch and intra-batch precision and accuracy of the assay for the H2 relaxin in rat plasma (n=5)

Spiked Concentration (ng/mL)	Intra-day			Inter-day		
	Determined concentration (Mean ± S.D., ng/mL)	R.S.D. (%)	R.E. (%)	Determined concentration (Mean ± S.D., ng/mL)	R.S.D. (%)	R.E. (%)
20.00	17.32±1.31	7.55	86.59	18.41±1.91	10.35	92.07
200.00	184.96±14.37	7.77	92.48	196.80±17.59	8.94	98.40
800.00	749.16±72.16	9.63	93.64	772.30±76.76	9.94	96.54



（A） （B）

Fig. 4 Representative peudo-MRM chromatograms of H2 relaxin and IS (A) a blank rat plasma; (B) a plasma sample 1h after s.c. of 0.5 mg/kg H2 relaxin.

Table 3 Pharmacokinetic parameters for H2 relaxin in rats after a single subcutaneous and intravenous dose of 0.5 mg/kg (Mean±S.D., n=6)

Parameters (Units)	s.c.	i.v.
t_{max} (h)	0.67±0.26	0.03±0.00
C_{max} (ng/mL)	191.49±64.94	940.20±60.57
AUC $_{0-t}$ (ng/mL*h)	325.51±93.78	373.98±130.94
AUC $_{0-\infty}$ (ng/mL*h)	376.90±99.13	395.70±135.25
$t_{1/2}$ (h)	1.23±0.32	0.97±0.48
CL (mL/min/kg)	23.95±8.63	23.15±7.70
V (mL/kg)	2456.78±639.96	1821.87±997.49
MRT $_{0-\infty}$ (h)	1.91±0.53	0.99±0.48
F (%)	95.20	

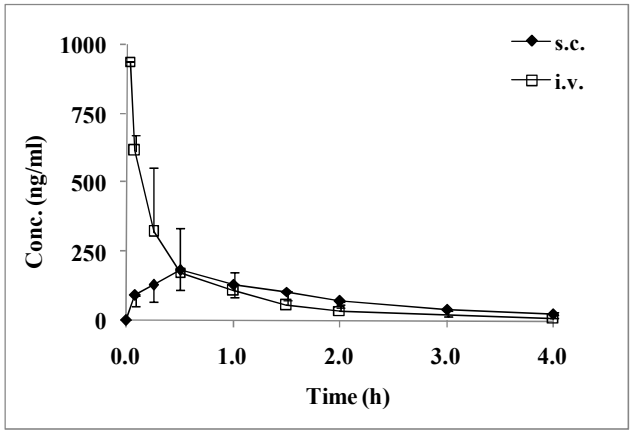


Fig. 5 Mean plasma concentration-time curves of H2 relaxin (Mean±S.D., n=6).

4. Conclusions

Overall, we have developed a simple, low cost, rapid and sensitive UPLC–MS/MS method to quantify H2 Relaxin in rat plasma samples for the first time. In spite of the complex biological matrixes, acceptable values of precision, accuracy and recovery were obtained. The data of rat PK with same dosage and administration route are consistent with those reported by Kraynov et al. using immunoassay method. The LC-MS/MS method will be applied for analysis of Recombinant Human Relaxin-2 (B29/A24) in pharmacokinetic and clinical studies to support investigational new drug application.

Acknowledgements

The project was supported by National High Technology Research and Development Program of China (863 Program) (No. 2002AA217031) and National Natural Science Foundation (Grant No. 30672464, 30772570, 30872393 and 81172973).

Notes and references

[a]*Minigene Pharmacy Laboratory, School of Life Science & Technology, China Pharmaceutical University, Nanjing, P.R. China; Tel: +86-25-83271346; Fax: +86-25-83271346; E-mail address: Taimingli@163.com (Taiming Li)*
[b]*Drug Metabolism and Pharmacokinetics Department, Shanghai Hengrui Pharmaceutical Co., Ltd., Shanghai, P.R. China*

1 J.D. Silvertown, A. Neschadim, H.N. Liu, P. Shannon, J.S. Walia, J.C. Kao, J. Robertson, A.J. Summerlee and J.A. Medin, *Regul. Pept.*, 2010, 159, 44-53.
2 E.T. van der Westhuizen, M.L. Halls., C.S. Samuel, R.A. Bathgate, E.N. Unemori, S.W. Sutton and R.J. Summers, *Drug Discov. Today*, 2008, 15, 640-651.
3 X.-L. Moore, S.-L. Tan, C.-Y. Lo, L. Fang, Y.-D. Su, X.-M. Gao, E.A. Woodcock, R.J. Summers, G.W. Tregear, R.A. Bathgate and X.-J. Du, *Endocrinology*, 2007, 148, 1582-1589.
4 D. Bani, *Gen. Pharmac*., 1997, 28, 13-22.
5 C.S. Samuel, T.D. Hewitson, E.N. Unemori and M.L. Tang., *Cell. Mol. Life Sci*., 2007, 64, 1539-1557.
6 S.L. Teichman, E. Unemori, T. Dschietzig, K. Conrad, A.A. Voors, J.R. Teerlink, G.M. Felker, M. Metra, G. Cotter, *Heart Fail Rev*., 2009, 14, 321-329.
7 V.B. Nair, C.S. Samuel, F. Separovic, M.A. Hossain and J.D. Wade, *Amino Acids*, 2012, 43, 1131-1140.
8 S.L. Teichman, E. Unemori, J.R. Teerlink, G. Cotter and M. Metra. *Curr. Heart Fail Rep*., 2010, 7, 75-82.
9 D.A. Parsell, J.Y. Mak, E.P. Amento and E.N. Unemori. *J. Biol. Chem*., 1996, 271 27936-27941.
10 V. Kraynov, N.Knudsen, A.Hewet, J.Pinkstaff, L.Sullivan and Kristine D. Dios, U.S. Patent, 2012, 0046229 A1.
11 D.L. Paccamonti, S.T. Chang, W. Dubois, C.M. Barros, M. Drost, C.J. Wilcox and M.J. Fields. *Theriogenology*, 1991, 35, 1131-1146.
12 H. Kosanam, S. Ramagiri and C. Dass. *Anal. Biochem*., 2009, 392, 83-89.
13 I. van den Broek, R.W. Sparidans, J.H. Schellens and J.H. Beijnen. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci*., 2008, 872, 1-22.
14 M. Ewles and L. Goodwin. *Bioanalysis*, 2011, 3, 1379-1397.
15 T. Klaassen, S. Szwandt, J.T. Kapron and A. Roemer. *Rapid Commun. Mass Spectrom*., 2009, 23, 2301-2306.
16 A. Thomas, W. Schänzer, P. Delahaut and M. Thevis. *Drug Test Anal*., 2009, 1, 219-227.
17 M. Thevis, A. Thomas and W. Schänzer. *Mass Spectrom. Rev*., 2008, 27, 35-50.