Journal of Chromatography A, 1216 (2009) 1490–1495

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Online preconcentration of recombinant Arg-Gly-Asp-hirudin using dynamic pH junction for analysis in human urine samples by capillary electrophoresis-mass spectrometry

Shengmin Su, Yunqiu Yu *

School of Pharmacy, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China

ARTICLE INFO

Article history:
Received 27 September 2008
Received in revised form
20 November 2008
Accepted 19 December 2008
Available online 25 December 2008

Keywords:
CE-MS
Online preconcentration
Dynamic pH junction
rRGD-hirudin
Human urine
Quantitative assay

ABSTRACT

A sensitive and effort-saving method was established and validated for the quantitative determination of recombinant Arg-Gly-Asp-hirudin (rRGD-hirudin) in human urine samples. The assay was performed on a uncoated fused silica capillary of 70 cm × 50 μm I.D. and a positive voltage of 30 kV was applied. The sample was injected under pressure of 50 mbar for 300 s and the temperature of capillary was kept 25 °C. Sheath liquid consisting of 30% methanol and 70% of 0.1% formic acid aqueous solution flowing at 7 μL/min was supplied to the CE-electrospray interface. Utilizing the dynamic pH junction technique, a lower limit of quantitation of approximately 35 nM was achieved (concentration coefficiency was about 100-fold) without complex sample preprocessing procedure. CE-MS conditions and parameters were also optimized to obtain better performance. The method has been successfully applied in clinical research of rRGD-hirudin.

1. Introduction

Hirudin, a powerful and specific thrombin inhibitor, is an anticoagulative product from salivary glands of medicinal leech. As direct thrombin inhibitor (DTI), hirudin represents a new class of therapeutics possessing theoretic advantages over unfractionated heparin (UFH). Compared to UFH, DTIs show several advantages such as non-activating platelets, having no circulating inhibitors, and binding to both free and clot-bound thrombin, which have spurred clinical trials investigating DTIs in a variety of cardiovascular indications [1]. Hirudin binds directly to thrombin, inhibiting fibrin bound as well as fluid-phase thrombin, while heparin which has to bind to anti-thrombin III to exert its actions. Hirudin also inhibits fibrinogen clotting and thrombin-catalyzed haemostatic reactions. Recombinant wild type (wt) hirudin is a single-chain, carbohydrate-free polypeptide containing 65–66 amino acids with a molecular weight of 7000 Da.

A novel bifunctional recombinant hirudin molecule, recombinant Arg-Gly-Asp-hirudin (rRGD-hirudin) was engineered by inserting the tripeptide RGD to a given domain of the wt-hirudin molecule [2]. In theory, rRGD-hirudin should inhibit both thrombin and platelet aggregation [3]. It is anticipated that rRGD-hirudin would compete heparin or wt-hirudin with higher efficacy and safety because of its bifunctional action and its low effective dosage.

So far, reported assays for determination of wt-hirudin in vivo mainly include activated partial thromboplastin time (aPTT) method [4,5], ecarin clotting time (ECT) method [6], chromogenic substrate assay (CSA) [7], enzyme-linked immunosorbent assay (ELISA) [8], radioimmunoassay (RIA) [9] and an isotope labeling tracer method [10]. Among these methods, aPPT and CSA are lack of specificity because they related to the level of thrombin or prothrombin which results in poor specificity and linearity because they can be influenced by other thrombin inhibitors such as heparin. While ECT is of better specificity but still will be strongly influenced by prothrombin level. Besides tedious sample preparation in ELISA and RIA, monoclonal antibody required is hard to prepare and of high cost. To overcome the deficiencies of the methods as previously described, a LC–MS method combining ultrafiltration and lyophilization for the determination of rRGD-hirudin in human serum has been developed and successfully applied in clinical trial in our laboratory [11]. Furthermore, we study on a CE method for the determination of the concentration of rRGD-hirudin in human urine, to investigate its pharmacokinetic property for it may be excreted mostly by kidney as reported earlier [12].

CE is widely utilized to bimolecular analysis due to its high efficiency and less solvent and sample consumption. With the developments of various online preconcentration techniques [13],

* Corresponding author. Tel.: +86 21 54237599.
E-mail address: yqyu@shmu.edu.cn (Y. Yu).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.12.050

both poor concentration sensitivity and low sample loading which limits CE applications in the past have been significantly improved. Among these techniques, dynamic pH junction was most suitable for the analysis of zwitterionic molecule such as peptides because its concentration mechanism is based on the significant changes in ionization states of the analytes or electrophoretic velocities between different pH values. In our study, rRDG-hirudin was dissolved in high pH buffer and injected to capillary filled with low-pH background electrolyte (BGE) as a long plug. When a positive voltage was applied, H^+ introduced from BGE titrated the high pH sample plug, converting anionic rRGD-hirudin molecule to cations and accumulated at boundary of BEG and sample zone. Besides, dynamic pH junction can afford sample containing high level of salt which makes it ideal for the analysis of bio-matrix samples comparing with other online preconcentration techniques such as field amplified sample stacking (FASS) or large volume sample stacking (LVSS) [14] which require sample in low conductivity matrix.

Dette reported a capillary zone electrophoresis (CZE) separation of hirudin and its similar substances [15]. Poly(ethylene glycol) of molecular weight 20 000 (PEG20000) and Zn^{2+} were added to electrolyte to achieve baseline separation of eight hirudin-related substances. Later, Dönges added multiple buffer additives to separate nine recombinant hirudin deviants [16]. These two researches demonstrated that determination of hirudin products by CE was possible, but the separation conditions must be well adjusted and neither of them took the detection limit issue into consideration. In Ban's study, r-hirudin was labeled with fluorescein isothiocyanate (FITC), and FITC-labeled r-hirudin was purified using high-performance liquid chromatography. The purified product was then mixed with the sample followed by the addition of anti-hirudin antibody. After that, free, antibody-bound, and tagged r-hirudin could be separated by CE with laser-induced fluorescence detection [17].

In this paper, we reported a sensitive and effort-saving assay for the determination of rRGD-hirudin in human urine sample. Separation was achieved without buffer additives and the lower limit of quantitation (LLOQ) was low at 0.25 μg mL^{-1} (~35 nM). Compared to the LLOQ of 20 nM in Ban's work, only a simple dilution process of sample was used in our method before the CE-MS run. Due to high online preconcentration efficiency, none of FITC labeling and LC purification were required. Clinical application result demonstrated that the assay was promising for urine-drug and therapeutical drug monitoring in the future.

2. Experimental

2.1. Chemicals and apparatus

Ammonium formate was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Deionized water was prepared using Direct-Q system from Millipore (Bedford, MA, USA) and pH value was monitored by 320 pH meter from Mettler Toledo. Uncoated fused silica capillary was purchased from Reafine Chromatography (Hebei, China).

CE-MS analysis was performed on an Agilent CE system and an Agilent G1946D mass-selective detector and an Agilent G1313A binary pump were also utilized to supply the sheath liquid for CE-electrospray ionization (ESI) MS interface.

2.2. Standard solution preparation

A stock solution was obtained by dissolving one ampoule of injection powder containing 5 mg of rRGD-hirudin with 10 mL deionized water. The stock solution was freshly prepared on every testing day.

A series of standard solutions was prepared by diluting the stock solution with different amounts of deionized water to give

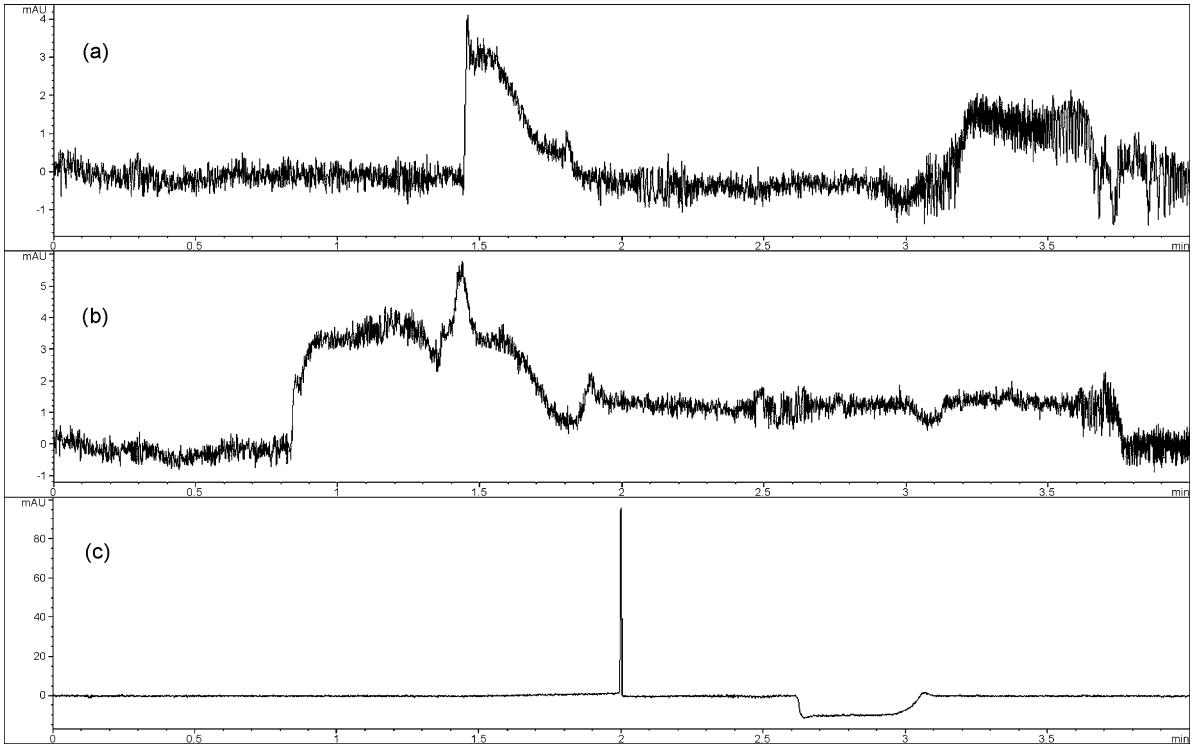

Fig. 1. Comparison between different injection time and dynamic pH junction. (a) Capillary: 30 cm × 50 μm I.D., BGE: 25 mM formic acid-ammonium formate (pH 2.4), sample in 25 mM formic acid–ammonium formate (pH 2.4), injection: 50 mbar × 30 s, UV: 200 nm. (b) Capillary: 30 cm × 50 μm I.D., BGE: 25 mM formic acid-ammonium formate (pH 2.4), sample in 25 mM formic acid-ammonium formate (pH 2.4), injection: 50 mbar × 120 s, UV: 200 nm. (c) Capillary: 30 cm × 50 μm I.D., BGE: 25 mM formic acid–ammonium formate (pH 2.4), sample in 25 mM formic acid–ammonium formate (pH 6), injection: 50 mbar × 30 s, UV: 200 nm.

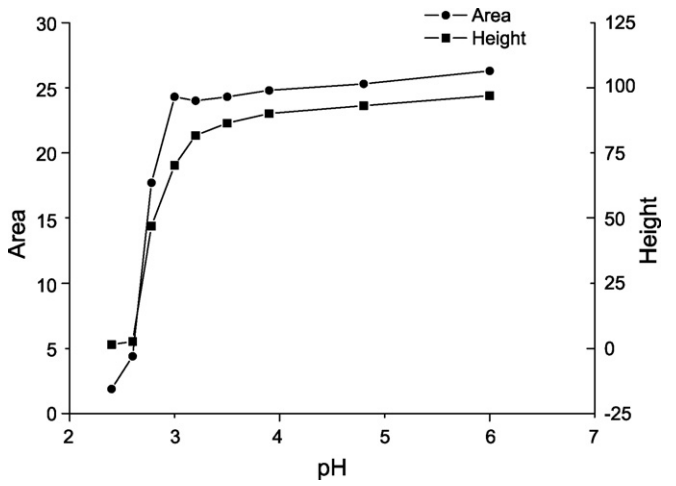

Fig. 2. The effect pH value of sample dilution solution on peak area and height of rRGD-hirudin.

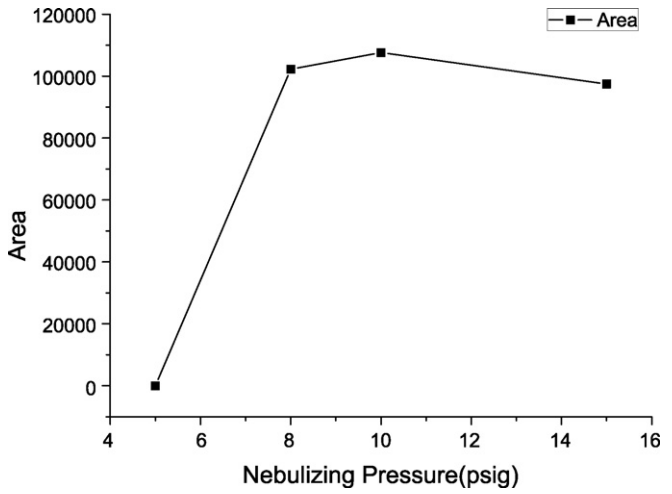

Fig. 4. The effect of nebulizing gas pressure on peak area of rRGD-hirudin. Drying gas flow rate: 3.5 L min^{-1}, drying gas temperature: 200 °C, sheath liquid consisted of 30% MeOH and 0.2% aqueous formic acid, flowing at 5 μL/min.

concentrations of 2.5 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, 20 μg mL^{-1}, 50 μg mL^{-1}, 100 μg mL^{-1} and 250 μg mL^{-1}, which were identified as standard solutions.

Urine-based calibration solutions were prepared by diluting the standard solutions with blank urine to yield final concentrations of 0.25 μg mL^{-1}, 0.5 μg mL^{-1}, 1 μg mL^{-1}, 2 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1} and 25 μg mL^{-1}. Three other standard solutions of 1.5 μg mL^{-1}, 6 μg mL^{-1} and 30 μg mL^{-1} were also prepared independently, as quality control samples, and were diluted to yield low (0.5 μg mL^{-1}), medium (2 μg mL^{-1}), and high (10 μg mL^{-1}) controls concentrations in human urine as quality control samples.

2.3. Sample preparation

100 μL of spiked urine samples and clinical samples were diluted by 900 μL of 25 mM ammonium formate aqueous solution (pH 6.0) followed by filtering through 0.22 μm pore size syringe filter.

2.4. CE-MS condition

CE BGE was prepared by titrating 25 mM ammonium formate aqueous solution (pH 6) to pH 2.4 with formic acid.

Uncoated fused silica capillary (70 cm × 365 μm O.D. × 50 μm I.D.) was flushed by deionized water for 15 min, 0.1 M HCl for 1 h, methanol for 1 h, 1 M NaOH for 1 h, successively, and rinsed by 0.1 M NaOH overnight before first use. Between every CE run, the capillary was flushed with BGE for 3 min.

After the sample was injected to capillary under pressure of 50 mbar for 300 s (concentration efficiency was approximately 100), a positive voltage of 30 kV was applied to start the CE run and the capillary was thermostated at 25 °C.

The CE-ESI interface was utilized to produce multi-charged molecules and the MS conditions were as follows: capillary voltage 5.5 kV; drying gas flow 3 L min^{-1}; drying gas temperature 350 °C; nebulizer gas pressure 10 psi. The MS detector was working in the selected ion monitoring (SIM) mode to monitor the rRGD-hirudin at m/z 1433, and the fragment voltage was set at 220 V [11].

Sheath liquid supplied by a LC binary pump consisted of 30% methanol and 70% of 0.1% formic acid aqueous solution. After a 99:1 splitting, the actual flow rate was 7 μL/min.

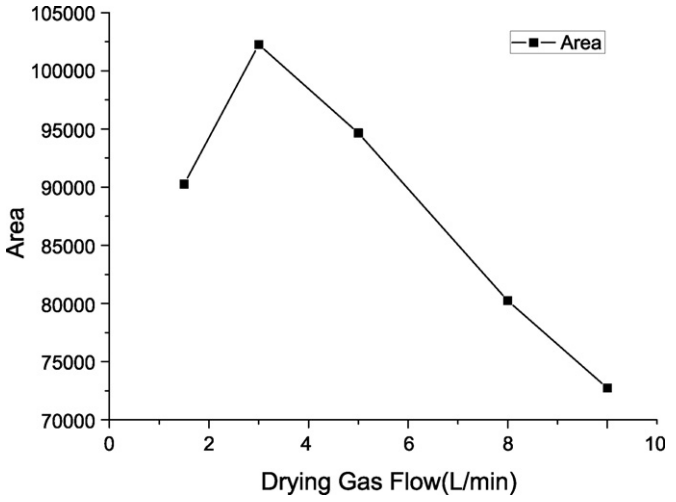

Fig. 3. The effect of drying gas flow rate on peak area of rRGD-hirudin. Nebulizing gas pressure: 8 psig, drying gas temperature: 200 °C, sheath liquid consisted of 30% MeOH and 0.2% aqueous formic acid, flowing at 5 μL/min.

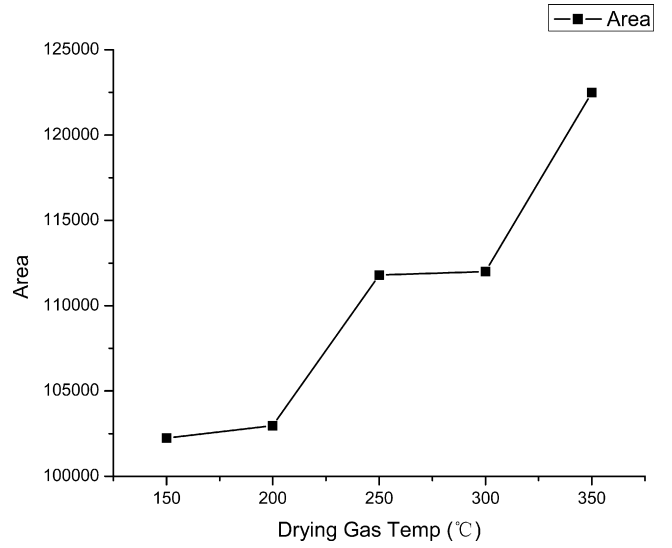

Fig. 5. The effect of drying gas temperature on peak area of rRGD-hirudin. Drying gas flow rate: 3.5 L min^{-1}, nebulizing gas pressure: 10 psig, sheath liquid consisted of 30% MeOH and 0.2% aqueous formic acid, flowing at 5 μL/min.

3. Results and Discussion

3.1. Optimization of dynamic pH junction conditions

Although the higher concentration of ammonium formate, the stronger buffering ability it would be, conductivity of BGE was also confined to the ESI chamber current upper limit which was restricted to 50 μA. After several tests, we found that 25 mM of ammonium formate in BGE and sample solution could meet the two requirements.

Considering that the isoelectric point (p*I*) of rRGD-hirudin was 4.1, it was beneficial to use acidic BGE both for MS detection and suppressing the absorption of rRGD-hirudin by uncoated fused silica capillary.

In our preliminary experiments, it was demonstrated that the detection limit of rRGD-hirudin cannot be improved by simply prolonging the injection time of sample, which would only lead to a non-stacking sample zone as shown in Fig. 1a and b and contrast to excellent stacking peak under dynamic pH junction mode Fig. 1c.

According to the theory of dynamic pH junction, pH value of sample zone was an important parameter of stacking and peak narrowing. A series of pH value ranging from 2.4 to 6 was tested to optimize the enrichment of rRGD-hirudin. As shown in Figs. 1 and 2, when the pH value was below 3, the CE condition was similar to CZE mode, so no obvious or only slight improvement could be observed. With the pH value of sample zone raised to 6, both peak area and height were increased significantly and sample zone was well stacked.

3.2. Optimization of CE-MS conditions

The ESI spray chamber conditions were optimized to give better ionization performance. The drying gas flow and nebulizer pressure were kept at relatively low level to match the low CE effluent combining with sheath liquid, while high drying gas temperature was helpful for desolvation as shown in Figs. 3–5.

The flow rate and composition of sheath liquid were well adjusted to maximum the MS signal response. Proper amount of sheath liquid could help maintain stable electrophoresis and electrospray without diluting the CE effluent, meanwhile, appropriate proportion of organic solvent and acid could facilitate the ionization procedure. The results are shown in Figs. 6–8.

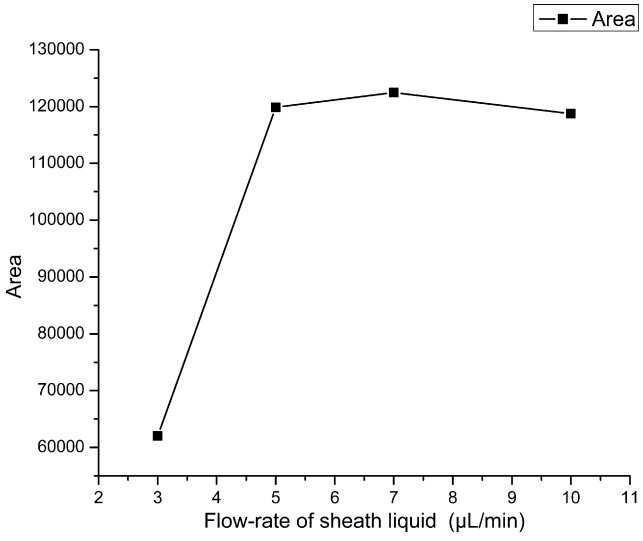

Fig. 6. The effect of sheath liquid flow rate on peak area of rRGD-hirudin. Drying gas flow rate: 3.5 L min^{-1}, nebulizing gas pressure: 10 psig, drying gas temperature: 350 °C sheath liquid consisted of 30% MeOH and 0.2% aqueous formic acid.

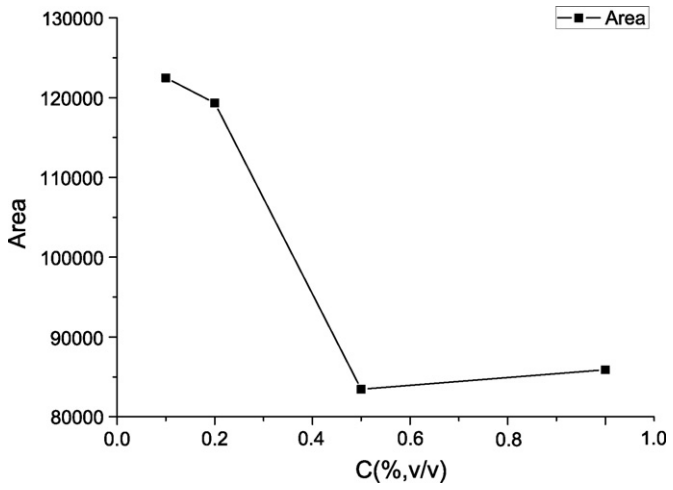

Fig. 7. The effect of formic acid concentration in sheath liquid on peak area of rRGD-hirudin. Drying gas flow rate: 3.5 L min^{-1}, nebulizing gas pressure: 10 psig, drying gas temperature: 350 °C sheath liquid consisted of 30% MeOH and aqueous formic acid, flowing at 7 μL/min.

3.3. Method validation result

3.3.1. Specificity

CE–MS electropherograms of rRGD-hirudin for a blank urine sample are shown in Fig. 9a, and a urine sample spiked with 2.5 μg mL^{-1} of rRGD-hirudin (0.25 μg mL^{-1} of rRGD-hirudin in urine) are shown in Fig. 9b. Typical electropherograms obtained from volunteer urine were shown in Fig. 9c. The electropherograms exhibited rRGD-hirudin could not be found in the blank urine. No additional peaks due to endogenous substances or other metabolite that could interfere with the detection of the compounds on the same retention time were observed.

3.3.2. Linearity and LLOQ

Due to the excellent stacking effect of dynamic pH junction, very sharp peak shape of rRGD-hirudin was obtained which made it possible to be quantitated both by peak area and peak height.

The calibration curve was constructed by plotting the area or height of rRGD-hirudin peaks as ordinate versus the concen-

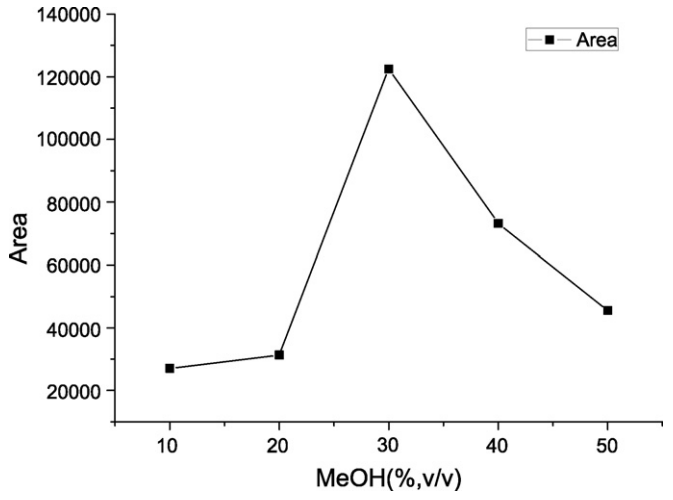

Fig. 8. The effect of MeOH concentration in sheath liquid on peak area of rRGD-hirudin. Drying gas flow rate: 3.5 L min^{-1}, nebulizing gas pressure: 10 psig, drying gas temperature: 350 °C sheath liquid consisted of MeOH and 0.1% aqueous formic acid, flowing at 7 μL/min.

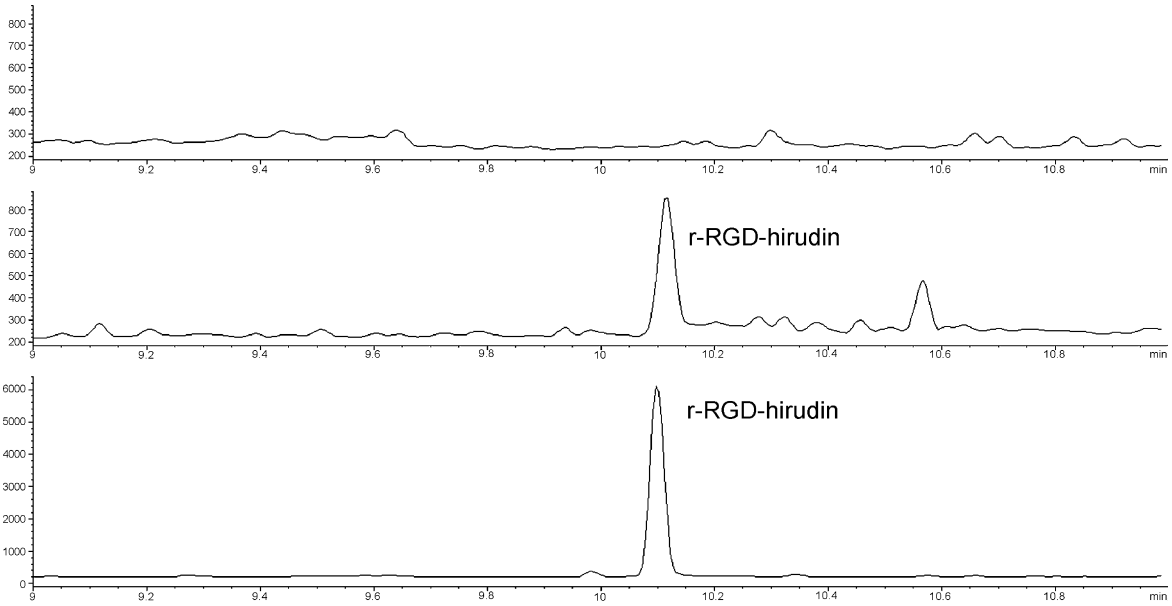

Fig. 9. The typical CE–MS electropherograms of rRGD-hirudin. (a) Blank urine sample; (b) spiked urine sample (0.25 μg mL^{-1} of rRGD-hirudin in urine); (c) volunteer urine sample. Capillary: 70 cm × 50 μm I.D., BGE: 25 mM formic acid–ammonium formate (pH 2.4), sample in 25 mM formic acid-ammonium formate (pH 6), injection: 50 mbar × 300 s, MS: m/z 1433 (SIM).

tration of rRGD-hirudin in human urine (μg mL^{-1}) as abscissa, $Y_{area} = 1818X + 165.1$ ($r = 0.9999$, $n = 7$); $Y_{height} = 1123X + 68.28$ ($r = 0.9999$, $n = 7$). The regression equation was generated by employing least square method. LLOQ was determined to be at 0.25 μg mL^{-1} with a signal-to-noise ratio of 10 to 1.

3.3.3. Accuracy precision and recovery

The intra-day accuracy and precision test were carried out at LLOQ and three typical concentrations in human urine (0.50 μg mL^{-1}, 2 μg mL^{-1}, and 10 μg mL^{-1}) by the analysis of five replicate samples; the inter-day test just repeated it on three successive days. The result calculated using peak area and height are shown in Tables 1 and 2, respectively.

Recovery efficiency was evaluated using three separate concentrations in urine (0.50 μg mL^{-1}, 2 μg mL^{-1}, and 10 μg mL^{-1}). The peak area of rRGD-hirudin obtained from spiked urine after prepossessed (described in Section 2.3) were compared to the peak area obtained from the nominal concentration of rRGD-hirudin. The recovery of the method was 80.25%, 82.73%, and 79.56% in three concentration levels.

3.3.4. Application in clinical trial

The tolerance test of rRGD-hirudin was carried out to evaluate the safety of the drug. Twenty-eight healthy volunteers were randomly divided into four dosage groups, and the determination of rRGD-hirudin was carried out earlier in our laboratory. Assuming that rRGD-hirudin would be mostly excreted through urine according to reported research, we applied the assay to one of the dosage groups (0.16 mg kg^{-1}) with the permission of the volunteers.

In 1-h time, rRGD-hirudin dissolved in 100 mL of normal saline was administrated to the volunteers through intravenous drip and urine samples were collected 1 h after the infusion was over.

As shown in Fig. 10, compared with blood level of rRGD-hirudin determined by LC–MS [11], the concentration in urine samples was 10–20-fold higher indicating that more attention should be paid to

Table 1
Intra- and inter-day RSD and accuracy for the determination of rRGD-hirudin in human serum using peak area.

Nominal concentration (μg mL^{-1})	Intra-day ($n = 5$)		Inter-day ($n = 3$)	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
0.25	102.93	6.13	105.17	14.49
0.5	96.56	10.74	97.92	17.98
2	96.79	3.45	103.43	20.06
10	102.66	4.41	102.52	15.15

Table 2
Intra- and inter-day RSD and accuracy for the determination of rRGD-hirudin in human serum using peak height.

Nominal concentration (μg mL^{-1})	Intra-day ($n = 5$)		Inter-day ($n = 3$)	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
0.25	102.86	4.30	108.65	8.58
0.5	97.18	6.70	100.50	14.59
2	97.42	2.21	104.50	15.99
10	102.84	2.92	103.42	12.23

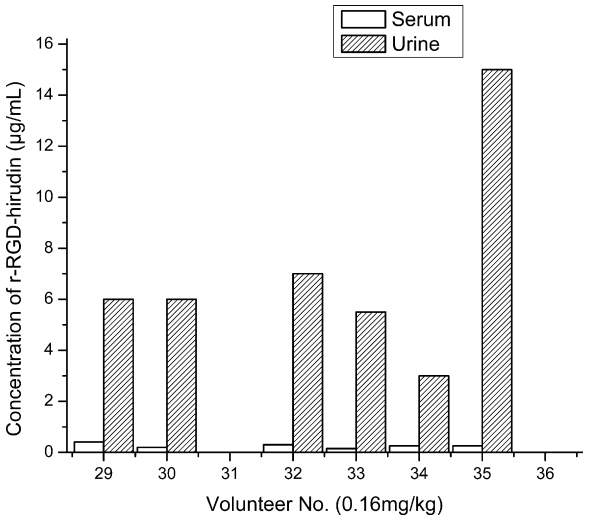

Fig. 10. Comparison of concentration of rRGD-hirudin in human serum and urine of volunteers. No. 31 and No. 36 volunteers were negative controls.

urine-drug method in further pharmacokinetic and pharmacodynamic study.

4. Discussion

The dynamic pH junction techniques applied in biological and pharmaceutical researches have been reported early [18–29]. Imami reported an application of dynamic pH junction CE-MS method to analyse tryptic digests of cytochrome *c in vitro*. A 50-fold sensitivity improvement was achieved in 15 min using strong acidic BGE (1 M formic acid) and sample matrix of high concentration of ammonium bicarbonate (100 mM) [21]. In our study, a more moderate CE condition was developed so as to decrease the capillary current which if too high (>50 μA) would probably cause damage to MS and also reduce the risk of denaturation of peptides. Compared to 50 $\mu g\,mL^{-1}$ for tryptic digests of cytochrome *c* (<1.5 kDa), our method quantitation limit was low to 0.25 $\mu g\,mL^{-1}$ *in vivo* for rRGD-hirudin (7 kDa). CE-ESI conditions were optimized in our study for better sensitivity.

5. Conclusion

A CE-MS method for the determination of rRGD-hirudin in human urine sample was developed and validated. Dynamic pH junction technique was adopted and optimized for the online preconcentration of the analyte to improve sensitivity. Due to the excellent concentration efficiency (~100-fold), conventional tedious sample preprocessing of bio-matrix sample was avoided and simple dilution process was used before CE-MS run. The parameter of CE-MS interface and ESI chamber were also optimized to obtain better MS signal response. The method has been successfully applied to real human urine samples collected during the tolerance test of rRGD-hirudin.

References

[1] M.S. Chen, A.M. Lincoff, Curr. Cardiol. Rep. 7 (2005) 255.
[2] W. Mo, Y.I. Zhang, L.S. Wang, X.Y. Yang, H.Y. Song, Thromb. Haemost. 93 (2005) A31.
[3] B. Adelman, A. Rizk, E. Hanners, Blood 72 (1988) 1530.
[4] A. Greinacher, H. Völpel, U. Janssens, V. Hach-Wunderle, B. Kemkes-Matthes, P. Eichler, H.G. Mueller-Velten, B. Pötzsch, Circulation 99 (1999) 73.
[5] F. Schiele, F. Lindgaerde, H. Eriksson, J.P. Bassand, A. Wallmark, P.O. Hansson, G. Grollier, M. Sjo, M. Moia, A. Camez, V. Smyth, M. Walker, Thromb. Haemost. 77 (1997) 834.
[6] B. Potzschl, S. Hund, K. Madlener, C. Unkrig, G. Muller-Berghaus, Thromb. Res. 86 (1997) 373.
[7] J.Y. Chang, FEBS Lett. 164 (1983) 307.
[8] X.H. Song, G. Huhle, L.C. Wang, J. Harenberg, Thromb. Res. 99 (2000) 195.
[9] K. Yasuhiko, H. Hideya, Thromb. Res. 87 (1997) 343.
[10] M.C. Wyers, M.D. Phaneuf, E.M. Rzucidlo, M.A. Contreras, F.W. LoGerfo, W.C. Quist, Cardiovasc. Pathol. 8 (1999) 153.
[11] S. Su, Y.Q. Yu, W. Mo, Y.L. Zhang, H.Y. Song, Q.F. Chen, Y. Xie, J. Chromatogr. B 870 (2008) 27.
[12] W. Raake, Haemostasis 21 (1991) 127.
[13] S.L. Simpson Jr., J.P. Quirino, S. Terabe, J. Chromatogr. A 1184 (2008) 504.
[14] C.-H. Lin, T. Kaneta, Electrophoresis 25 (2004) 4058.
[15] C. Dette, H. Wfitzig, J. Chromatogr. A 700 (1995) 89.
[16] R. Dönges, D. Brazel, J. Chromatogr. A 979 (2002) 217.
[17] E. Ban, H.-S. Nam, Y.S. Yoo, J. Chromatogr. A 924 (2001) 337.
[18] J.B. Kim, Y. Okamoto, S. Terabe, J. Chromatogr. A 1018 (2003) 251.
[19] Z.Q. Xu, T. Nishine, A. Arai, T. Hirokawa, Electrophoresis 25 (2004) 3875.
[20] A.S. Ptolemy, M. Le Bilhan, P. Britz-McKibbin, Electrophoresis 26 (2005) 4206.
[21] K. Imami, M.R.N. Monton, Y. Ishihama, S. Terabe, J. Chromatogr. A 1148 (2007) 250.
[22] M.R.N. Monton, K. Imami, M. Nakanishi, J.B. Kim, S. Terabe, J. Chromatogr. A 1079 (2005) 266.
[23] A.S. Ptolemy, P. Britz-McKibbin, Analyst 130 (2005) 1263.
[24] S.H. Shim, A. Riaz, K.W. Choi, D.S. Chung, Electrophoresis 24 (2003) 1603.
[25] P. Britz-McKibbin, T. Nishioka, S. Terabe, Anal. Sci. 19 (2003) 99.
[26] S.R. Mei, C.H. Yao, L.S. Cai, J. Xing, G.W. Xu, C.Y. Wu, Electrophoresis 24 (2003) 1411.
[27] P. Britz-McKibbin, D.D.Y. Chen, Chromatographia 57 (2003) 87.
[28] L.Y. Fan, L.H. Liu, H.L. Chen, X.G. Chen, Z.D. Hu, J. Chromatogr. A 1062 (2005) 133.
[29] G. Manetto, F. Tagliaro, F. Crivellente, V.L. Pascali, M. Marigo, Electrophoresis 21 (2000) 2891.