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A development of LC–MS method combining ultrafiltration and lyophilization for determination of r-RGD-Hirudin in human serum

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ABSTRACT

A reliable and validated LC–MS method was established for determination of r-RGD-Hirudin in human serum. Ultrafiltration was used instead of liquid–liquid extraction or solid phase extraction for water solubility drug r-RGD-Hirudin extraction. Freeze drying was used for concentration. The experiment conditions, including pre-processing procedure and LC–MS, have been investigated and optimized. Comparing with reported assays, the current method showed significant improvement in specificity, linearity, precision and sensitivity. This method has been successfully applied in clinical research of r-RGD-Hirudin.

1. Introduction

Hirudin is an anticoagulative product from salivary glands of medicinal leech. It is a powerful and specific Thrombin inhibitor. As direct Thrombin inhibitors (DTIs), Hirudin is a new class of therapeutics possessing theoretic advantages over unfractionated heparin (UFH). In contrast to UFH, DTIs do not activate platelets, have no circulating inhibitors, and bind to both free and clot-bound Thrombin. These theoretical advantages have spurred clinical trials investigating DTIs in a variety of cardiovascular indications [1]. Unlike heparin, which has to bind to anti-Thrombin III to exert its actions, Hirudin binds directly to Thrombin, inhibiting Fibrin-bound as well as fluid-phase Thrombin. It also inhibits Fibrinogen clotting and Thrombin-catalysed haemostatic reactions.

Recombinant wt-Hirudin is a single-chain, carbohydrate-free polypeptide containing 65–66 amino acids with a molecular weight of 7000 Da. Wt-Hirudin has a trisulfide-linked core structure, forming a compact NH_2-terminal domain and a long COOH-terminal extended domain. The crystallographic structure of Hirudin–Thrombin complex showed abundant interactions of these two molecules, both polar and apolar, which may account for the tight affinity and specificity of Hirudin over that of heparin.

Adhesion of platelets to vessel walls, their activation, and aggregation is a central primary event in blood coagulation. On activated platelets, the glycoprotein IIb/IIIa (GP IIb/IIIa) functions as a receptor for Fibrinogen to mediate platelet aggregation and crosslinking. The capability of GP IIb/IIIa to bind to Fibrinogen and other adhesive proteins is due to its ability to recognize the Arg-Gly-Asp (RGD) motif within their sequences. Peptides containing the RGD sequence or tripeptide RGD crossed out alone competes and inhibits the binding of Fibrinogen to GP IIb/IIIa on platelets, thus inhibiting platelet aggregation. Adelman proved that a peptide with an internal RGD sequence remarkably prevented platelet aggregation induced by ADP [2].

A novel bi-functional recombinant Hirudin molecule, r-RGD-Hirudin, were engineered by inserting a tripeptide RGD to a given domain of the wt-Hirudin molecular [3]. In theory, r-RGD-Hirudin should inhibit both Thrombin and platelet aggregation. It is anticipated that r-RGD-Hirudin would compete heparin or wt-Hirudin with higher efficacy and safety because of its bi-functional action and its low effective dosage.

The current reported assay 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 related to

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Table 1
Quantitative parameter in LC–MS method compared with reported methods to determine Hirudin in vivo

	LC–MS method	ELISA [8]	ECT [6]	aPTT [4,5]
Linearity (r)	0.99	0.86	0.9	Not linear, but hyperbolic
LLOQ (ng mL^{-1})	25	100	50	
Precision (RSD,%)	All below 15%	Mostly above 15%		
Specificity	Good	Poor	Fair	
Analytical period	Rapid	Tedious		

level of Thrombin or Prothrombin, are widely used in determination of different kinds of Thrombin inhibitors like heparin, so they lack specificity; the snake venom enzyme Ecarin converts Prothrombin to Meizothrombin which can be rapidly neutralized by Hirudin resulting in a dose-dependent prolongation of the clotting times, but it was also reported that the ECT method can be strongly influenced by the Prothrombin level [11,12]. While ELISA and RIA require a monoclonal antibody, which is of higher cost and hard to prepare, the isotope labeling tracer required cannot be used in human body.

Chromatographic methods, especially LC–MS has been found suitable for drug monitoring in body fluids such as plasma and serum. In order to determine small quantity of substances in those complicated biological matrices, various kinds of sample preprocessing procedure is always utilized prior to chromatographic system.

However, because of its high water solubility, r-RGD-Hirudin cannot be efficiently extracted by organic solvent partitioning. Conventional extraction methods, such as liquid–liquid extraction and solid phase extraction, are not useful for r-RGD-Hirudin. Precipitation treatments will denaturate the polypeptide and the analysis fails too.

To overcome the deficiencies of those methods as previously described, a novel LC–MS method combining ultrafiltration and freeze drying was established in my laboratory. The experimental conditions including pre-processing procedures and LC–MS have been investigated and optimized. The pre-processing procedure includes ultrafiltration, which was used in separating r-RGD-Hirudin from serum, and freeze drying, which was used to concentrate the drug to improve detection outcome. MS detector working on selective ion monitoring mode ensured the specific detection of the r-RGD-Hirudin without interference of Thrombin, Prothrombin or other anticoagulation drugs. Comparing with aPTT, ECT, and ELISA (Table 1), demonstrated that our method can provide much better linearity, and precision at a lower lower limit of quantification (LLOQ) which make it a more satisfactory assay in a clinical trial and therapeutic drug monitoring of r-RGD-Hirudin. The method has been successfully applied in clinical research of r-RGD-Hirudin.

2. Experimental

2.1. Chemicals and apparatus

Organic reagents such as methanol and formic acid were purchased from Dikma (40 Vogell Road, Unit 57, Richmond Hill, ON, L4B 3N6); Microcon YM-30K, YM-50K and YM-100K centrifugal filter devices were purchased from Millipore (Bedford, MA 01730, USA)

r-RGD-Hirudin was offered from the key laboratory of molecular medicine (Fudan University).

LC–MS analysis was performed using Agilent (Palo Alto, USA) 1100 series system, which consisted of a G1322A degasser, a G1312A binary pump, a G1313A plus autosampler, a G1316A column compartment with thermostat, and a G1946D quadrupole MS detector. Data were collected and analyzed on an Agilent G2710AA analysis workstation. The separation was performed using Zorbax Extended-C18 (150 mm × 2.1 mm × 5 μm, ID), connected with a security guard cartridge (C18, 4 mm × 3.0 mm, Phenomenex USA). The Alpha2-4 freeze dryer (Martin Christ Gemany) and TJ-25 centrifuge (Beckman USA) was used in the sample preparation procedure.

2.2. Standard solution preparation

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

A series of standard solutions were prepared by diluting the stock solution with different amount of deionized water to give concentrations of 250 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1}, 2000 ng mL^{-1}, 5000 ng mL^{-1}, 10,000 ng mL^{-1} and 25,000 ng mL^{-1}, which were identified as standard solutions. Serum-based calibration solutions were prepared by using the dilution standard solution procedure to yield final concentrations of 25 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 200 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1} and 2500 ng mL^{-1}.

Three other standard solutions of 150 ng mL^{-1}, 600 ng mL^{-1} and 3000 ng mL^{-1} were also prepared, independently, as quality control samples, and were diluted to yield low (50 ng mL^{-1}), medium (200 ng mL^{-1}), and high (1000 ng mL^{-1}) controls concentrations in human serum as quality control samples.

2.3. Sample preparation

The pre-processing of the spiked serum samples or clinical samples included three major steps: first, the 150 μL of serum were diluted with 300 μL of 20% (v/v) formic acid; second, the diluted serum were transferred to a Microcon ultrafiltration tube and centrifuged at 12,000 × g for 20 min under room temperature, after the filtrate was collected, the centrifuge was repeated once more and the filtrates were put together into an Eppendorf tube and frozen at −70 °C for 1 h; third, a freeze drying method was used to evaporate the solvent in the filtrate and leave the r-RGD-Hirudin in the tube for the assay determination.

The residue was reconstituted with 50 μL of 0.1% (v/v) formic acid for 15 min before the LC–MS analysis.

2.4. LC–MS conditions

2.4.1. HPLC condition

10 μL of reconstituted sample was injected into an Agilent Extended-C18 column. The initial mobile phase gradient (flow rate 0.3 mL/min) consisted of 70% 0.1% formic acid (A) and 30% methanol (B). The HPLC gradient was gradually increased from 30% methanol to 65% in 6 min followed by a rapid increasing to 100% in 1 min to flush the column. After that, it took 8 min for the column to equilibrate to the initial condition.

2.4.2. MS conditions

Electrospray ionization source (ESI) was utilized to produce multi-charged molecules and conditions of MS detector were as follows: capillary voltage, 5.5 kV; source temperature, 350 °C; drying gas flow, 13 L min^{-1}; fragment voltage, 220 V; nebulizer pressure, 55 psi.

2.5. Method validation

2.5.1. Specificity

Specificity was determined by comparing the blank serum sample with both spiked serum sample and serum of volunteers who received the injection of r-RGD-Hirudin to assess potential interference from endogenous substances.

2.5.2. Linearity and LLOQ

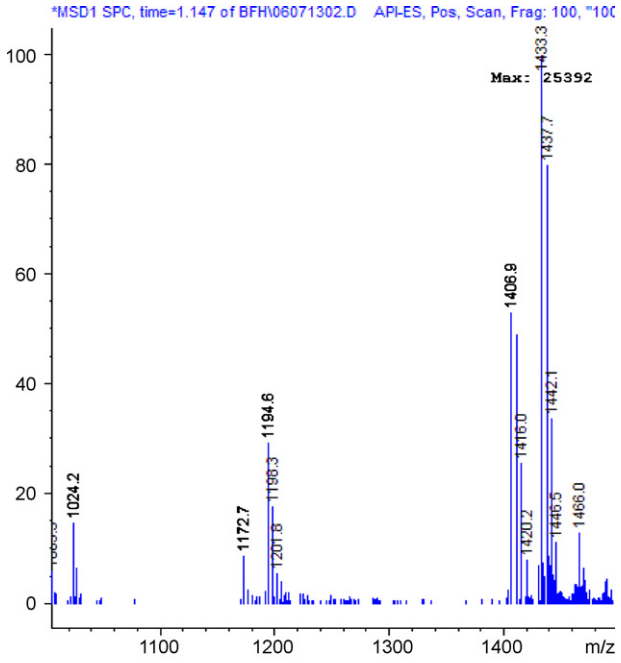
The linearity was determined by injecting several serum-based calibration samples (25 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 200 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1} and 2500 ng mL^{-1} in blank human serum) according to the sample preparation procedure described above.

LLOQ was the lowest standard on the calibration curves which was 25 ng mL^{-1}.

2.5.3. Accuracy precision and recovery

The intra-day accuracy and precision test were carried out at LLOQ and three typical concentrations in human serum (50 ng mL^{-1}, 200 ng mL^{-1}, and 1000 ng mL^{-1}) by analysis of five replicate samples; the inter-day test just repeated it on three successive days.

Recovery efficiency was evaluated using three separate concentrations in serum (50 ng mL^{-1}, 200 ng mL^{-1}, and 1000 ng mL^{-1}). The peak area of r-RGD-Hirudin obtained from spiked serum after pre-possessed (described in Section 2.3) were compared to the peak area obtained from the nominal concentration of r-RGD-Hirudin.



m/z 1024.2 [r-RGD-Hirudin+7H$^+$]$^{7+}$
m/z 1172.7 [des-Glu r-RGD-Hirudin+7H$^+$]$^{7+}$
m/z 1194.6 [r-RGD-Hirudin+6H$^+$]$^{6+}$
m/z 1198.3 [r-RGD-Hirudin+5H$^+$+Na$^+$]$^{6+}$
m/z 1201.8 [r-RGD-Hirudin+4H$^+$+2Na$^+$]$^{6+}$
m/z 1406.9 [des-Glu r-RGD-Hirudin+5H$^+$]$^{5+}$
m/z 1416.0 [des-Glu r-RGD-Hirudin+3H$^+$+2Na$^+$]$^{5+}$
m/z 1420.2 [des-Glu r-RGD-Hirudin+2H$^+$+3Na$^+$]$^{5+}$
m/z 1433.3 [r-RGD-Hirudin+5H$^+$]$^{5+}$
m/z 1437.7 [r-RGD-Hirudin+4H$^+$Na$^+$]$^{5+}$
m/z 1442.1 [r-RGD-Hirudin+3H$^+$2Na$^+$]$^{5+}$
m/z 1446.5 [r-RGD-Hirudin+2H$^+$3Na$^+$]$^{5+}$

Fig. 1. Full scan mass spectra of r-RGD-Hirudin.

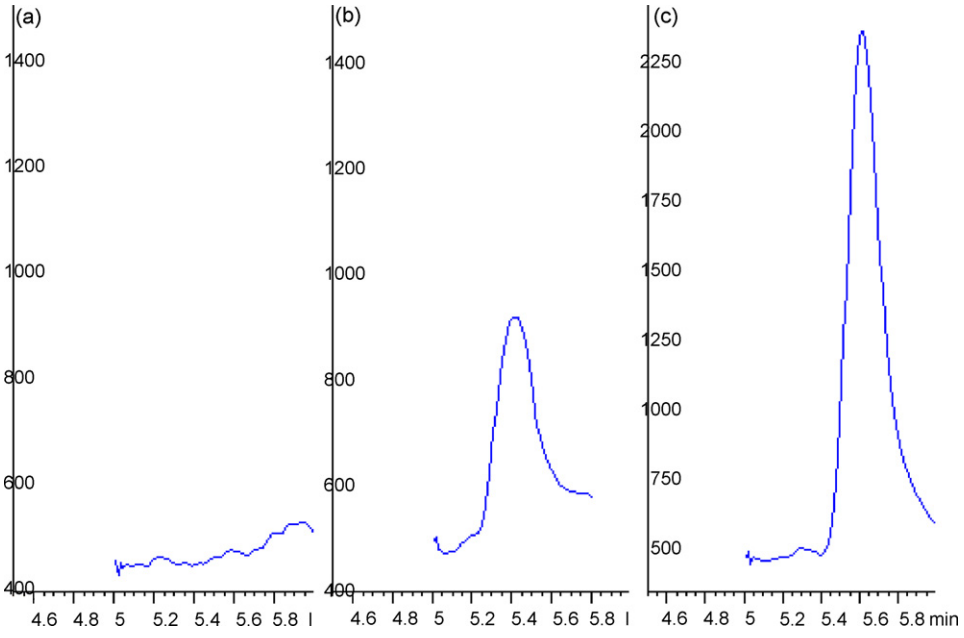


Fig. 2. Chromatograms monitored on *m*/*z* 1433 of (a) blank serum, (b) spiked serum sample, and (c) volunteer serum sample.

3. Result

3.1. Method validation result

3.1.1. Specificity

In the ESI spectrum for r-RGD-Hirudin (Fig. 1), 5–7 positive charge molecular species are observed at m/z 1433.3 (+5), 1194.6 (+6) and 1024.2 (+7). In addition a des-Glu species is observed at m/z 1406.9. The +5 charged molecular ion at m/z 1433 was used for specific detection and quantification.

LC–MS chromatograms of r-RGD-Hirudin for a blank serum sample were shown in Fig. 2(a), and a serum sample spiked with 250 ng mL^{-1} of r-RGD-Hirudin (25 ng mL^{-1} of r-RGD-Hirudin in serum) were shown in Fig. 2(b). Typical chromatograms obtained from volunteer serum were shown in Fig. 2(c). The chromatograms exhibited r-RGD-Hirudin could not be found in the drug-free serum. 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.

3.1.2. Linearity and LLOQ

The calibration curve was constructed by plotting the area of r-RGD-Hirudin as ordinate versus the concentration of r-RGD-Hirudin in human serum (ng mL^{-1}) as abscissa, $Y = 115.99X - 252.09$ ($r = 0.9993$), while the regression equation was generated by employing least square method. LLOQ was determined to be at 25 ng mL^{-1} with a signal to noise ratio of ten to one.

3.1.3. Accuracy precision and recovery

Table 2 result showed that assay meets the method validation regulation. The recovery of the method was 61.35%, 75.77%, and 73.43%, respectively in three concentration levels ($n = 5$).

3.2. Application in clinical trial

The tolerance test of r-RGD-Hirudin was carried out to evaluate the safety of the drug. Twenty-eight healthy volunteers were randomly divided into four dosage groups (0.04 mg kg^{-1}, 0.08 mg kg^{-1}, 0.12 mg kg^{-1} and 0.16 mg kg^{-1}); six in each of the first two groups and eight in each of other two group, while in each group there were two volunteers that actually received normal saline to give a negative control. In 1 h time, r-RGD-Hirudin dissolved in 100 mL of normal saline were administrated to the volunteers through intravenous drip and serum was collected 1 h after the infusion was over.

Fig. 3, with the increasing concentration of r-RGD-Hirudin and prolongation of aPTT and TT with the raising dosage indicated a positive correlation of dosage and effect.

Table 2
Intra- and inter-day coefficient of variation and accuracy for determination of r-RGD-Hirudin in human serum

Nominal C (ng mL^{-1})	Intra-day ($n = 5$)		Inter-day ($n = 3$)	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
25	104.27	12.00	103.40	15.80
50	96.62	12.99	97.41	8.19
200	98.40	4.14	96.33	8.06
1000	100.07	7.26	97.55	14.50

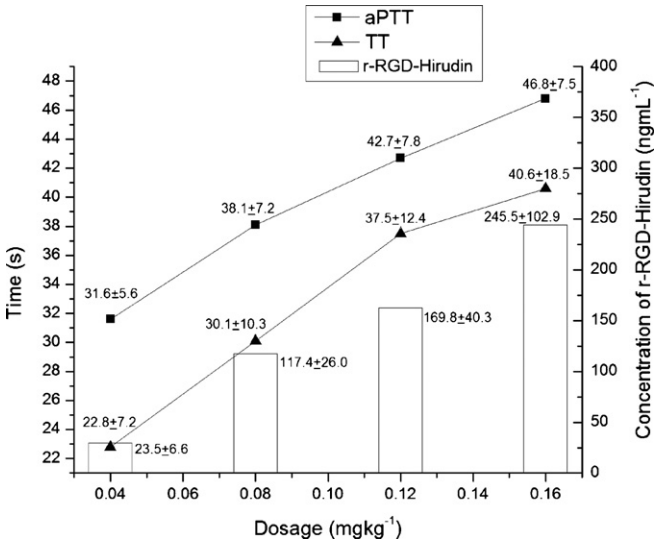


Fig. 3. Tolerance test result.

4. Discussion

4.1. Optimization of sample pre-processing procedure

In the diluting step, several concentrations of formic acid were tested to investigate the acidity effect on ultrafiltration ratio. The ultrafiltration ratio was calculated by comparing the chromatographic peak area of r-RGD-Hirudin after ultrafiltration versus the standard solution. As shown in Fig. 4, the ultrafiltration ratio increased with the raising concentration of formic acid. r-RGD-Hirudin binds irreversibly to the Thrombin molecule at the catalytic site (via its globular amino-terminal domain) and to exosite 1 (via its carboxy-terminal domain) and forms a slowly reversible complex [13,14]. This reversible complex can separate in acidic solution. With the raising concentration of formic acid, r-RGD-Hirudin dissociates to Thrombin, which resulted in the raising ultrafiltration ratio. So, the concentration of formic acid was set to 20% (v/v) to be used to dilute the serum.

Three different types of Microcon ultrafiltration tubes, YM-30K, YM-50K and YM-100K were tested. The chromatographic peak area was as an index to evaluate the ratio of filtration of Microcon ultra-

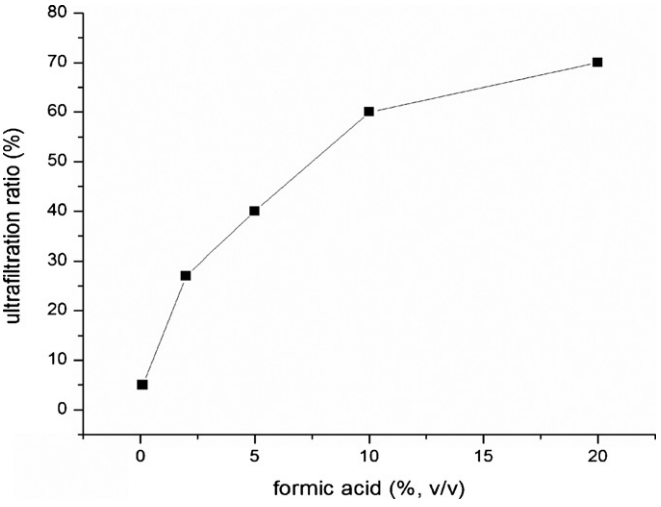


Fig. 4. The effect of formic acid concentration on ultrafiltration rate.

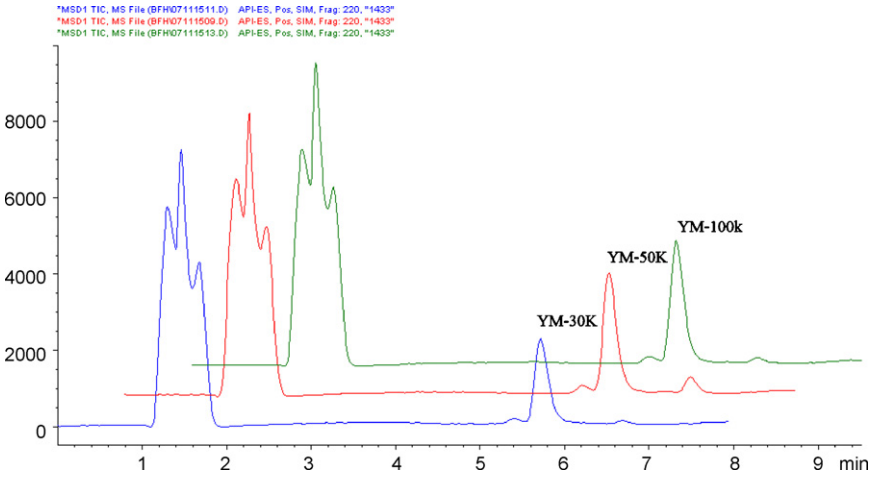


Fig. 5. The effect of three different Microcon ultrafiltration tube YM-30K, YM-50K and YM-100K on peak area of r-RGD-Hirudin.

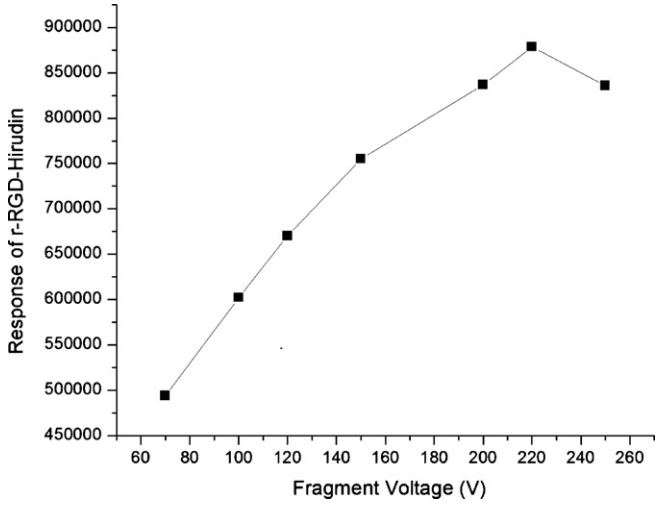


Fig. 6. The effect of fragment voltage on response of r-RGD-Hirudin.

filtration tube. As shown in Fig. 5, the peak area of ultrafiltration by using YM-30K was smaller than that for YM-50K and YM-100K, indicating the amount of r-RGD-Hirudin in filtrate of YM-30K tube was less than that of YM-50K and YM-100K. In further investigation, the YM-30K Microcon tube failed because the LLOQ cannot be lower than 50 ng mL^{-1} and the accuracy and precision were also poor; not only showing no advantage in yield compared to YM-50K Microcon tube, but the YM-100K Microcon tube would also potentially allow in many more macromolecules to the filtrate from serum likely to caused the deterioration of separation column. Therefore, YM-50K Micron tube was found to be highest recovery and fewest impurities.

4.2. Optimization of LC–MS condition

Flow injection analysis (FIA) was employed to determine the best MS source condition. As shown in Fig. 6, it seemed that the fragment voltage greatly influence the MS signal response. When it was increased from 70 V to 220 V, the signal response of r-RGD-Hirudin also demonstrated a significant heightening, but when continued increasing to 250 V, the response drop instead. Considering that fragment voltage was applied between the transmission capillary and the skimmer, we assume that higher voltage can facilitate the transfer of multi-charged r-RGD-Hirudin molecules coming from the ion source to the mass analyzer, so the response goes up, became too high, the macromolecules began to decompose resulting in fewer intact molecules to be detected.

5. Conclusion

A LC–MS method was established to determine the r-RGD-Hirudin in human serum. Ultrafiltration was employed for separation and purification of r-RGD-Hirudin and the freeze drying was employed for concentration. Our new method shows excellent specificity and sensitivity when compared with the commonly used analytical methods such as aPTT, ECT, CSA, ELISA, and RIA for r-RGD-Hirudin analysis in vivo. We also optimized experimental conditions and validated the method of its precision, accuracy, sensitivity and linearity. The method has been successfully used in clinical investigation on tolerance test of r-RGD-Hirudin.

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