ORIGINAL ARTICLE

LC-MS/MS ANALYSIS FOR PEPTIDES USING HUMAN BRADYKININ AND TWO OF ITS FRAGMENTS AS MODEL MOLECULES

VALENTIN ION, SILVIA IMRE*, ANCA-GABRIELA CARJE, ALINA BALINT, DANIELA-LUCIA MUNTEAN

University of Medicine and and Pharmacy, Faculty of Pharmacy, Department of Analytical Chemistry and Drug Analysis, 38 Gheorghe Marinescu Street, 540139, Târgu Mureș, Romania

**corresponding author: silsta@yahoo.com*

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Abstract

Peptide analysis in the context of targeted or discovery proteomics applications assisted by liquid chromatography coupled with mass spectrometry gained increasingly more interest in the last ten years. In any of the proteomics applications cases, an efficient separation of the analysed peptides alongside the chromatographic column is foreseen. That is why this article aimed offer a better insight of peptide chromatographic and mass spectrometric behaviour. As model molecules, human bradykinin and two of its fragments, Bradykinin 1-8 and Bradykinin 1-7 were selected. A reversed phase-mode liquid chromatography-triple quadrupole-mass spectrometry analytical method was developed, investigating the influence of the elution gradient slope of the mobile phase organic component and chromatographic column temperature influence, over the method selectivity α. This two studied components proved to be critical in respect with method selectivity and analytes separations. As a step forward, a fast and simple sample preparation method was developed for the analysis of the three peptides in human urine, overall sample preparation-liquid chromatographic-QQQ method complying with general bioanalytical validation parameters and thresholds.

Rezumat

Analiza peptidelor în contextul studiilor de proteomică asistate de cromatografia de lichide cuplată cu spectrometria de masă a câștigat tot mai mult interes din partea cercetătorilor în ultimii ani. Separarea eficientă a peptidelor de-a lungul coloanei este prevăzută în oricare direcție de cercetare din proteomică - *discovery proteomics* sau *targeted proteomics* (proteomică la țintă). De aceea, acest articol își propune să ofere o mai bună aprofundare asupra comportamentului cromatografic și de ionizare-fragmentare-detecție prin spectrometria de masă a peptidelor. Bradikinina umană și două dintre fragmentele sale, bradikinina 1-8 și bradikinina 1-7, au fost selectate drept molecule model. A fost dezvoltată o metodă analitică HPLC în fază inversă cuplată cu spectrometrie de masă de tip triplu cvadrupol, investigând pe rând, influența pantei gradientului de eluție și influența temperaturii coloanei asupra selectivității metodei cromatografice. Aceste două componente studiate s-au dovedit a fi de importanță critică cu privire la performanța metodei cromatografice. Suplimentar, a fost dezvoltată o metodă simplă și rapidă de prelucrare a probelor în vederea analizării celor trei peptide în urina umană. Ansamblul metodei de prelucrare a probelor și metodei de analiză lichid cromatografică cuplată cu spectrometria de masă, s-a dovedit a corespunde cu limitele impuse de parametri generali de validare bioanalitică.

Keywords: LC-MS/MS, bradykinins, peptides, bioanalytical validation

Introduction

In the last five or ten years, one can observe a growing interest in the field of targeted or discovery proteomics with its applications in clinical chemistry. Liquid chromatography coupled with mass spectrometry (LC-MS, LC-MS/MS-tandem) turned to be a powerful tool for the demands of targeted or discovery proteomics. LC-MS/MS technique offers high selectivity, on one part with the help of liquid chromatography as an analytical separation method and on the other part the mass spectrometry (MS) which, nowadays attains and reaches high accuracy and precision in mass over charge ratios. Far beyond targeted proteomics, due to the improvements in MS technology, structural elucidation and confirmation for unknown compounds now can be possible [2, 5, 11].

For targeted proteomics applications solved with the help of LC-MS/MS, very often, tryptic digestion must forego LC-MS/MS analysis. On other words, proteins are digested into small peptides, some of them being used as proteotypic peptides or signature peptides for the targeted protein. The signature peptides must be unique and specific for the protein from which they are derived from [12].

It is already known that peptides exhibit different chromatographic behaviour when analysed in reversed phase mode (RP-LC) compared to small molecules; and is considered erroneous to admit that their separation in RP-mode is based on continuous

partition between the stationary phase and the mobile phase [6]. It is more likely to say that peptides adsorb to the stationary phase and they are eluted only when a critical percentage in organic component of the mobile phase is reached. At that critical moment, the organic modifier reaching a certain percentage will disrupt the hydrophobic interactions between the peptides and stationary phase. On the other hand, chromatographic temperature might be a critical parameter even for absorbtion-desorbtion mechanisms and might have influence over the analysis time improvement, peak shape and selectivity [3]. Even though sample preparation methods for biological samples containing peptidic analytes are highly specific (example-solid phase extraction, immunodepletion etc.) sometimes, some much more simple methods such as dilution, protein precipitation and centrifugation might be used for peptide analysis in relatively simple biological matrices like human urine [4].

The aim of our study was to give a better insight over peptide chromatographic behaviour, studying the influence of the gradient elution slope of the organic modifier over the method selectivity and chromatographic temperature contribution to selectivity improvement. To achieve the aims of this study, three peptides were chosen, namely human bradykinin (B 1-9) and two of its fragments, bradykinin 1-8 (B 1-8) and bradykinin 1-9 (B 1-9). The choice of this peptides is not random. On one hand, chemical structural similarity was pursued and on the other, a moderate extended interval in terms of peptide molecular weights was foreseen [1].

Furthermore, a step forward was made, trying to develop a simple sample preparation method for the analysis of this three compounds in human urine, investigating if the overall sample preparation - LC-MS/MS process comply with the main analytical validation parameters. This study was undertaken for a better understanding of the matrix effect over the analytical method performance.

Materials and Methods

Chemicals and standards

Pure water provided by a Millipore Q5 (Millipore Q-Merck) system was used for samples and mobile phase preparations. Acetonitrile (ACN), liquid chromatography grade, used for sample and mobile phase preparations was purchased from VWR (VWR International GmbH). Methanol (MeOH) liquid chromatography grade was purchased from VWR (VWR International GmbH). Formic acid (FA), LC-MS grade, was purchased from Fluka and was used for the sample preparations and as mobile phase additive. Trifluoracetic acid (TFA), LC-MS grade, was acquired from Sigma Aldrich and was used for sample preparations.

Human bradykinin (in the form of Bradykinin acetate - B 1-9) and bradykinin fragments, bradykinin 1-8 (bradykinin 1-8 acetate - B1-8) and bradykinin 1-7 (B 1-7) were purchased from Sigma Aldrich. All three peptides used in this study were used as standards of high analytical purity. B 1-9 was accurately weighted (Mettler Toledo analytical balance) and dissolved in a mix solvent (MeOH/Water = 50/50, v/v) to provide a stock solution of 1 mg/mL. The stock solution was further diluted to 100 µg/mL, this final solution being aliquoted and stored to freezer at -50ºC (Sanyo Freezer). B 1-8 and B1-7 underwent the same procedures with the specification that the samples were prepared in 100 mM formic acid. All standard/stock solutions were prepared with the help of Eppendorf Protein LoBind type tubes and Eppendorf LoRetention (epT.I.P.S®) pipette tips.

Liquid Cromatography and Mass Spectrometry apparatus

An Agilent 1100 Series High Performance Liquid Chromatography system (Agilent Technologies-USA) was used. The system was equipped with a quaternary pump, a mobile phase degasser, a thermostated column compartment, a thermostated autosampler and an UV-VIS detector. The UV-VIS detector was bypassed to avoid dead volumes. The chromatographic investigations were conducted with the help of a Zorbax © Extend column with a C_{18} RP doubly end-capped stationary phase (Agilent Technologies USA). The column was filled with 3.5 µm particles with 80 Å pore size. The column length was 100 mm. Preceding the column, a Zorbax 300StableBond-C_{18} (Agilent Technologies) BioLC guard column was used (300 Å pore size). Guard Column was used to protect the chromatographic column and to allow the analytes completely access to the stationary phase.

Mass spectrometric parameters were established and chosen using an infusion pump (kdScientific) and infusion syringe (Agilent Technologies), prior to LC method development. Mass spectrometric detection was made possible with the help of an Agilent 6410 Series Triple Quadrupole (QQQ) Mass Spectrometer (Agilent Technologies USA) equipped with an electro-spray ionisation source (ESI) working in positive mode (ESI+).

MS/MS detection parameters and LC-MS method development

Multiple reactions monitoring (MRM) is a typical scan type mode for triple quadrupole mass spectrometers, providing high specificity for the detection of the targeted analytes, being also a suitable tool for chemical structure elucidation. Prior to MS infusion with the help of the infusion pump, each peptide was further diluted up to a final concentration of 1 µg/mL with the help of a suitable solvent. The solvent had the following composition: ACN/FA

0.1% = 15/85 (v/v). This solvent was chosen to provide similarity with the mobile phase as respect to ionisation conditions.

Ionisation source parameters were the same for all three analytes and were set as follows: Positive ionisation mode; Capillary Voltage – 4500 V (+), Ionisation source temperature – 350ºC; Dry Gas – 12 L/min, Nebuliser – 25 Psi.

Chosen MRM pattern for human bradykinin (B 1-9) followed the transitions: 531.0 m/z → 531.0 m/z and 531.0 m/z → 70.0 m/z. For Bradykinin 1-8, the following transition was adopted: 452.8 m/z → 642.3 m/z. Bradykinin 1-7 exhibited subsequent specific transitions: 379.2 m/z → 642.2 m/z and 379.2 m/z → 555.2 m/z.

For the peptides separation, a gradient elution mode was selected. Mobile phase was composed of two types of solvents, each one on a separate pump line. Solvent A consisted of 0.1% FA (v/v) prepared in ultrapure LC grade water. Solvent B, predominantly organic, was composed of ACN/Water = 90/10 (v/v) with an overall addition of 0.1% FA (v/v). Prior to LC separation, each of the solvent A and B was ultrasonicated using an ultrasonic bath. Mobile phase flow through the column was set at 0.8 mL/min under an initial gradient elution mode, as presented in Table I.

Table I

Initial elution gradient

Gradient Step	Minute (min.)	Solvent B (%)
First Part-Linear Increase	0	10
	6.66	32
Second Part	6.8	90
	9.0	90
Column Re - equilibration	9.10	10
Post Time - Re-equilibration	+ 2.0	10

This initial gradient underwent changes as respect to gradient slope in the first part-linear step of the gradient. Column compartment temperature was varied between 20ºC and 45ºC to observe the influence of the separation temperature over the method selectivity. To avoid as much as possible ionisation source contamination, mobile phase bypassed the MS after exiting the column, being redirected to waste before the elution of the first analyte and after the elution of the last analyte. Autosampler was thermostated at 7ºC to ensure and provide analyte chemical stability all along the analysis intervals. Each time, autosampler was set to inject on column 2 µL sample volume.

Sample preparation methods

All the samples destined for LC-MS/MS analysis were prepared using Eppendorf LoBind tubes and Eppendorf LoRetention pipette tips. It is already known that peptides exhibit nonspecific adsorption on hydrophobic surfaces due to their hydrophobic side-chains. Moreover, for this reason, silanised glass inserts were used for sample storage inside LC vials [8, 9].

For the first part of the study, more exactly, the studies regarding the influence of gradient slope and temperature over selectivity, samples were prepared as neat solutions using ACN/0.1% FA = 15/85 (v/v) as solvent. Each injected solution was prepared containing all three peptides at a concentration level of 1 µg/mL or 500 ng/mL each.

In the second part of the study, pooled human urine was used. The samples came from the sample bank where were stored at -70ºC. Human urine collection procedure complied with the declaration of Helsinki. Thawed urine was acidified with absolute TFA to give a 2% concentration in TFA. Acid addition step aimed to interrupt all enzymatic activities that might occur by sharply lowering the pH. Afterwards, acidified urine was spiked with 100 µg/mL B 1-9, B 1-8 and B 1-7 to give a starting concentration of 2 µg/mL for each peptide in acidified urine. This spiked biological stark sample was further diluted with blank acidified urine up to the targeted concentrations. Spiked biological samples were afterwards treated with ice-cold ACN (-20ºC) as follows: to 100 µL spiked biological sample, 400 µL ice cold ACN was added for protein and salts precipitation and for sample dilution. Further on, samples were mixed for 10 seconds with the help of a vortex device. Afterwards, samples were centrifuged at 13,000 RPM for 10 minutes at 7ºC using a refrigerated centrifuge (Sigma). After centrifugation, a suitable supernatant volume was collected and transferred into LC vials provided with silanized inserts. LC vials were kept inside the autosampler at 7ºC prior to injection. QC standards were prepared in neat solvents, taking into account a dilution factor of five, under which biological samples were subjected during sample preparation procedures.

Analytical validation parameters

Main validation parameters were taken into account for the method validation investigation: limit of quantification (LOQ), specificity, carry over, linearity, accuracy, precision. Limit of quantification was established as the lowest concentration where signal

to noise ratio (S/N) had a value of 10 or slightly higher and the coefficient of variation (CV%) at the lowest quantification level was less than 20%. Specificity was checked by injecting blank acidified urine sample that undergone sample preparation [7].
Linearity was verified over an interval of concentrations comprised between 20 ng/mL and 400 ng/mL for each of the three peptides using five concentration levels. Moreover, linearity was checked both for biological samples and neat solutions samples for the same concentration levels (QC), for each level, three replicates being submitted. Accuracy was considered to comply with, when recovery (Re%) (relative ratio between analytical response for biological samples and analytical response for neat solutions) was comprised between 85% and 115%. Within-day-precision was evaluated with the help of the CV% generated by three successive injections replicates, at all five investigated levels of concentrations.

Results and Discussion

MS/MS fragmentation pattern for the targeted peptides
Peptide fragmentation pattern at the collision cell level is not random, being well established. Often, fragmentation might occur at the level of the peptide main amino-acid chain, where peptide bonds are created between two amino-acids. Charge retention will occur either on the part of the molecule containing the intact carboxyl-terminal group or amino-terminal function. Thus being said, 2 main types of daughter ions can be generated: a, b, c series with charge retention on the N-terminus, this series being numbered from the amino terminus and x, y, z series with charge retention on C-terminus, this series being numbered from the carboxyl group [13].
Even though peptides fragment usually by generating "y" ions, B 1-9, B 1-8 and B1-7 behave typically, generating predominantly "b" type ions as showed in Table II.

Table II
MS/MS peptide fragmentation patterns and parameters

Peptide amino-acid Sequence	Precursor ion m/z (charge)	Fragment ions m/z (charge)	Generated Fragment ion	Fragmentation parameters	
B 1-9 *R*PPGFSPFR	531.0 (+2)	531.0 (+2)/ 70.0 (+2)	- b_1-NH_3	Dwell	100/100
				Collision Energy (V)	20/55
				Fragmentor (V)	135/175
B 1-8 *RPPGFSP*F	452.8 (+2)	642.3 (+1)	b_6	Dwell	100
				Collision Energy (V)	11
				Fragmentor (V)	135
B 1-7 *RPPGFS*P	379.2 (+2)	642.3 (+1)/ 555.2 (+1)	b_6 b_5	Dwell	100/100
				Collision Energy (V)	5/5
				Fragmentor (V)	110/110

Influence of the linear gradient slope against selectivity
Selectivity α is defined as the ratio between capacity factor K_a of the analyte of interest and capacity factor K_r of the reference analyte. In our experiments, at a constant chromatographic column temperature of 45ºC, a mix solution containing all the peptides at a concentration value of 1 µg/mL was injected. To observe the influence of the linear gradient slope over the selectivity, elution slope in the first part of the gradient was modified. By keeping the same starting/ending percentage of Solvent B (starting - 10%/ending - 32%) for the first part of the gradient, slope was modified by ramping up the speed of solvent B from 3.33% increase/min to 4.33% increase/min; 5.33% increase/min and finally 6.33% increase/min respectively. For the different slope values, selectivity α was computed for B 1-9 and B1-8; B1-7 being used as reference peak compound.

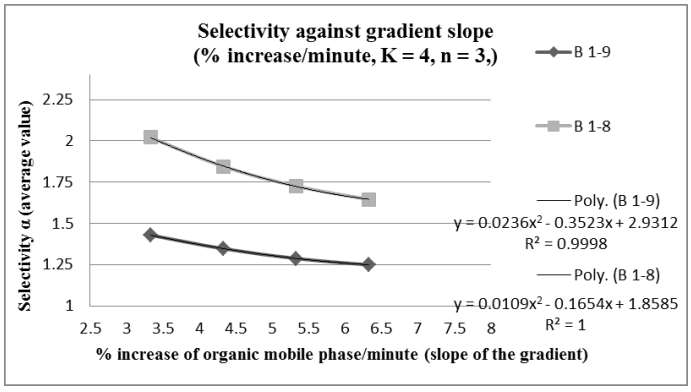

Figure 1.
Selectivity α against the slope of the linear gradient

As can be seen in Figure 1, both B 1-8 and B 1-9 selectivities decrease in a quadratic manner as the linear gradient slope increases. At a 6.33%/min increase in solvent B, Selectivity α for both B 1-9 and B 1-8 comply with a minimum accepted standard value of 1.2. Moreover, under this elution slope rate, overall time analysis was reduced at 7 minutes + 2 minutes post time for column equilibration.

Temperature influence over the method selectivity

Chromatographic separation temperature is a critical parameter regarding chromatographic method performance. Temperature might have an impact over method selectivity, peak width and shape, but also peak resolutions. The impact of the column temperature over selectivity α was investigated by varying column temperature using a 6.33%/minute linear increase in solvent B in the first part of elution gradient. Column temperature was varied between 45ºC and 20ºC and, as respect to Figure 2. Selectivity α for both B 1-9 and B 1-8 linearly increases with column temperature. To fully confirm that selectivity statistically linearly increases with column temperature, F statistical test was used to check if the two slopes obtained by plotting the selectivity against temperature are statistically significant. Experimental F values were greater than theoretical F values ($p < 0.05$) meaning that the two slopes are significant, otherwise said that, selectivity for both B 1-8 and B 1-9 statistically increases with the increase of column temperature under a linear dependence.

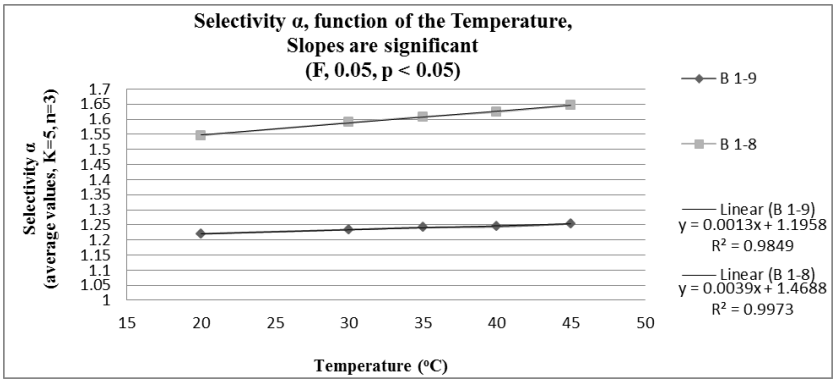

Figure 2.

Selectivity α against the column compartment temperature

BioAnalytical Method Performance

To further develop the bioanalytical method, column temperature was set at 45ºC and a gradient slope linear increase in Solvent B of 6.33%/min was kept. By using this method parameters, overall analysis time decreased to seven minutes adding two supplementary post time minutes for column equilibration between consecutive injections. All three peptides were successfully base-line solved with the following average retention times and elution order: B 1-7 – 3.55 min. (n = 15, SD = 0.006); B1-9 – 4.07 min. (n = 15, SD = 0.005); B 1-8 – 4.89 min (n = 15, SD = 0.003).

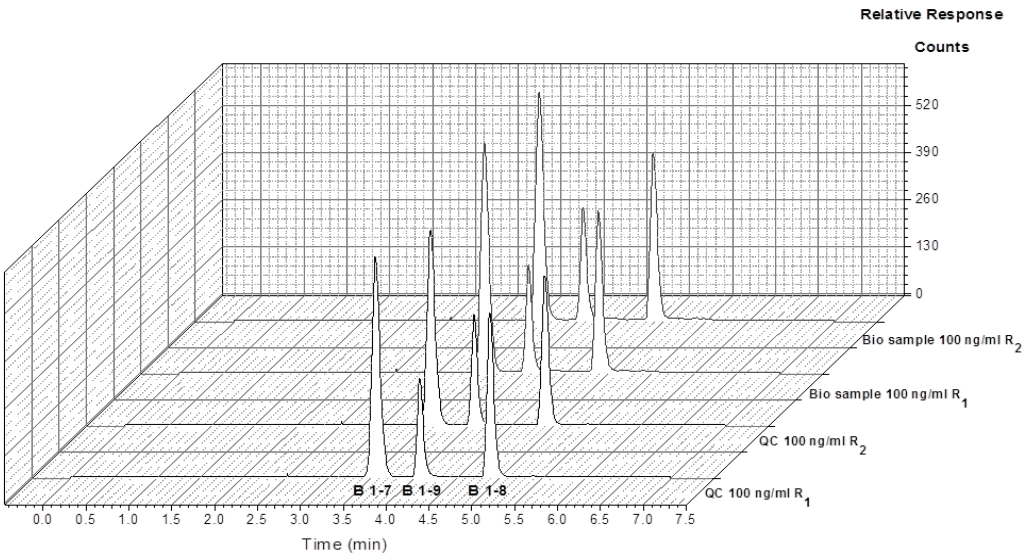

Figure 3.

Typical chromatograms at a 100 ng/mL concentration level

Limit of quantification

Calculated LOQ concentrations were set at a S/N ratio of 10 and a CV% of less than 20% as shown in Table III.

Table III

LOQ concentration levels for B 1-9; B 1-8; B 1-7

Peptide	LOQ (ng/mL) Neat solutions & Injected biological samples	CV%	LOQ (ng/mL) Unprocessed spiked urine samples
Human Bradykinin B 1-9	10	7.15%	50
Bradykinin Fragment B 1-8	2	10.72%	10
Bradykinin Fragment B 1-7	1	5.7%	5

Specificity and carry-over

Specificity was verified by injecting a blank/un-spiked urine sample. No peak was found at the corresponding retention times of B 1-7, B 1-9 and B 1-8. Carry-over was assessed by successively injecting three replicates of biological samples at the highest concentration level of 400 ng/mL. Consecutively, a blank neat solution was injected to verify if peaks will appear at the corresponding retention times. No peak appeared at the corresponding retention times for all the three peptides.

Linearity

Linearity was verified at five concentration levels for all three peptides, both for neat solution samples (QC standards) and biological samples as follows: 20 ng/mL; 40 ng/mL; 100 ng/mL; 200 ng/mL; 400 ng/ml. For each concentration level, 3 replicates were injected/tested. The linear calibration model was confirmed with the help of the computed determination coefficient R^2 and residuals analysis. All the residuals were within 85% - 115% limit. A 1/x weighting factor fit the model.

Table IV

Calibration curves parameters for neat and biological samples

Peptide	Tested dynamic range	Average calibration curves equations for QC neat samples	R^{2-} calibration curves for QC neat solutions	Average calibration curves equations for biological samples	R^2-calibration curves for biological samples
B 1-9	20 ng/mL	y = 15.682x - 175.27	R² = 0.993	y = 15.28x - 65.027	R² = 0.999
B 1-8	-	y = 29.928x - 69.483	R² = 0.999	y = 27.021x - 17.547	R² = 0.999
B 1-7	400 ng/mL	y = 50.949x - 192.65	R² = 0.998	y = 49.215x - 343.88	R² = 0.999

Accuracy and Precision

Accuracy and precision were tested for the same concentration levels as for linearity. The method is accurate and precise, as the recovery percentages vary between 88.28% and 113.7% and CVs% vary between 0.02% and 10.1% respectively. Peptides are situated between small molecules and proteins if molecular weights and chemical structure are taken into consideration. Nevertheless, considering this sample preparation procedures namely-protein precipitation and sample dilution (followed by centrifugation) and observing recovery percentages for the biological samples (Table V), one might have a better insight over the fact that peptides do not precipitate alongside with urinary residual proteins and salts. On the other hand, literature reports cases in which peptides tend to be non-specifically adsorbed at the surface of the deposited protein pellet thereby influencing overall peptide recovery. Usually, sample preparation methods for biological peptides/proteins found in biological samples like urine, plasma, serum are much more specific (solid phase extraction, immunoaffinity depletion of large proteins, SDS-gel electrophoresis etc.) due to the matrix effect of high abundant molecules such as lipids or abundant proteins (albumins) [11]. On the other hand, one cannot neglect the dual behaviour of biological peptides, the function of their chemical structure or chosen sample preparation method and the phenomenon the might occur under basic sample manipulations.

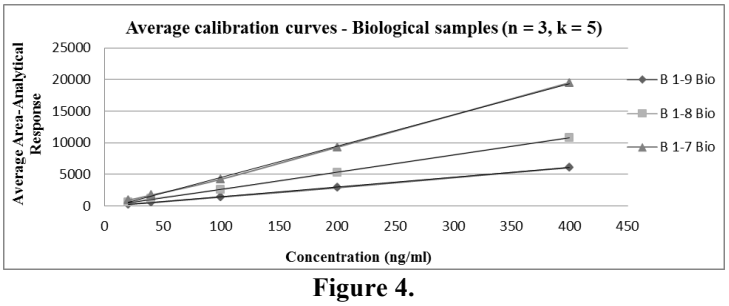

Figure 4.

Average calibration curves for biological samples

Table V

Accuracy and precision – mean recovery values and CVs% (k = 5, n = 3)

Peptide	Concentration Level (ng/mL)	Recovery (Re%) (Average)	CV%
B 1-9	20	88.28	4.02
B 1-8		93.30	10.1
B 1-7		90.82	0.60
B 1-9	40	110.2	8.2
B 1-8		92.10	0.43
B 1-7		85.30	1.60
B 1-9	100	113.70	0.90
B 1-8		97.72	0.02
B 1-7		99.27	1.25
B 1-9	200	108.35	1.92
B 1-8		88.77	2.16
B 1-7		90.49	3.70
B 1-9	400	97.40	0.43
B 1-8		90.95	0.72
B 1-7		96.65	3.08

Conclusions

In complex analytical samples, where LC-MS/MS assists targeted proteomics applications, targeted protein is subjected to in-solution digestion generating signature peptides (bottom-up proteomics). On the other hand, in the case of discovery proteomics, where all proteome variations is under strict observation, it is necessary to appeal to protein fractionation, protein separation and isolation with the help of on-gel electrophoresis; prior to a final protein digestion before LC-MS/MS analysis. Either way, for the two main types of proteomics applications assisted by LC-MS/MS, generated peptides are separated alongside the chromatographic column and further analysed with the help of mass spectrometer. It is hereby unmistakable that an effective separation for as many peptides as possible it is desirable, avoiding thus co-elution. Further on, an effective ionisation and detection at the level of mass spectrometer means improving method analysis sensibility, compound discovery and efficient protein structural elucidation. Column temperature and linear gradient slope have an impact over peptide separation performance and can be used as critical separation parameters in a factorial design when separation performance, selectivity or resolution does not comply, for different peptide separation applications.
Moreover, the three compounds were able to be analysed in a simple biological matrix like human urine, using a fast non-specific sample preparation method and complying with the main analytical method parameters and thresholds. This simple method can be used for the analysis and investigation of the kallikrein-kinin system by measuring bradykinin and its fragments or bradykinin degradation rate in human urine.

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