Method Development for the Quantitation of a Urine Biomarker for Acute Kidney Injury Using a QTRAP® mass spectrometer

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INTRODUCTION

The use of mass spectrometry for the detection and quantitation of protein biomarkers has many advantages in comparison to antibody based quantitative analysis (ELISA). The specificity of detection in regards to protein sequences is very high, even different protein isoforms can be selectively analyzed. Whereas ELISA quantitation are dependent on antibody production and quality, mass spectrometry is an unbiased method and showed therefore higher reproducibility. Finally mass spectrometry offers the possibility of high multiplexing, many different proteins can be analyzed in one single run. Here we present a study for the development of a method for the detection and quantitation of neutrophil gelatinase-associated lipocalin (NGAL) in human urine by mass spectrometry using a QTRAP® 6500 system. NGAL is discussed as an important inflammatory biomarker in research into renal function.

MATERIALS AND METHODS

Sample Preparation: Neutrophil gelatinase-associated lipocalin (NGAL), was purchased from Sigma Aldrich (St. Luis, USA). 10 µg of NGAL was digested with trypsin after reduction and alkylation to give a standard digest for MRM method development. Different ways of sample preparation for NGAL quantitation in urine were tested in a second step, spiking urine with increasing concentrations of NGAL (50 ng/ml, 100 ng/ml, 250 ng/ml and 500 ng/ml). Three methods for protein purification were tested with regard to the reproducibility of quantitation and recovery of the potential biomarker: Protein precipitation with ice cold methanol, protein purification using centrifugal filter devices and size exclusion clean-up spin-columns. Every urine sample was split into three equal portions for every sample preparation method to determine the reproducibility of the whole process. The protein purification steps were followed by reduction and alkylation of all cysteines with DDT and Iodoacetamide and tryptic digestion. Peptide quantitation of selected NGAL peptides by MRM /MS3 was performed on a 6500 QTRAP® system (AB SCIEX, Framingham, USA) .

HPLC Conditions: For micro flow separation, an Eksigent ekspert™ microLC 200 system (AB SCIEX, Framingham, USA) with an Eksigent HALO™ C18, 2.7 µm, 90Å, 0.5 x 100 mm at 40˚ C with a gradient of 15 min gradient was used at a flow rate of 25 µL/min. The injection volume was set to 5 µL and 10 µl (samples from spin column desalting).

MS/MS Conditions: For this study, an AB SCIEX QTRAP® 6500 system was used. MRM method development was performed using MIDAS™ Workflow experiments (MRM triggered MS/MS experiments) to confirm the peptide sequence and optimize MRM transitions. For quantification, MRM and MRM^3 workflows were performed.

Data Processing: The protein identification data was processed using ProteinPilot® and MultiQuant™ Software to perform quantification and statistical analysis. Skyline software (University of Washington was used for MRM development.

RESULTS

MRM / MS3 method development

A MRM method for the quantitation of NGAL in Urine was developed using the Skyline Software (MacCoss Lab Software). MRM transitions were predicted for all tryptic peptides from NGAL, excluding peptides, which contain methionine in the sequence. Predicted transitions were tested using MIDAS™ workflow experiments (MRM triggered MS/MS experiments) on the QTRAP® 6500 system. All MSMS spectra were searched against the Uniprot/SwissProt database for verification and used in addition by the Skyline software to select the most intensive and selective fragments for MRM. In a last step collision energies for the selected transitions were optimized.

Q1	Q3	Sequence.Charge state.Fragment	CE
597.63	836.39	VPLQQNFQDNQFQGK.+3y.7	24.1
597.63	721.36	VPLQQNFQDNQFQGK.+3y.6	24.1
597.63	680.37	VPLQQNFQDNQFQGK.+3b.6	27.1
716.41	983.6	WYVVGLAGNAILR.+2.y10	37.6
716.41	884.53	WYVVGLAGNAILR.+2.y9	34.6
716.41	714.43	WYVVGLAGNAILR.+2.y7	28.6
593.32	935.53	SYNVTSVLFR.+2.y8	27.2
593.32	821.49	SYNVTSVLFR.+2.y7	30.2
593.32	722.42	SYNVTSVLFR.+2.y6	27.2
456.71	752.37	C[+57.0]DYWIR.+2.y5	25.3
456.71	637.35	C[+57.0]DYWIR.+2.y4	25.3
456.71	474.28	C[+57.0]DYWIR.+2.y3	25.3
628.34	908.52	SYPGLTSYLVR.+2.y8	37.5
628.34	738.41	SYPGLTSYLVR.+2.y6	37.5
628.34	637.37	SYPGLTSYLVR.+2.y5	37.5
361.71	609.34	ITLYGR.+2.y5	18.9
361.71	508.29	ITLYGR.+2.y4	15.9
361.71	395.2	ITLYGR.+2.y3	18.9
410.23	690.4	ELTSELK.+2.y6	23.6
410.23	577.32	ELTSELK.+2.y5	20.6
410.23	476.27	ELTSELK.+2.y4	20.6

Table 1: MRM table of selected NGAL peptides.

Shown here is the table for all selected NGAL peptides and fragments together with the optimized collision energy on the QTRAP® 6500 system

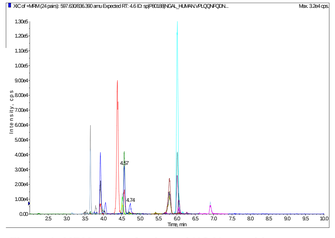


Figure 1: MRM trace of selected NGAL peptides.

XIC for all transitions from selected NGAL peptides. Example trace is from a 50 ng/ml spike of NGAL in Urine, after Amicon purification, reduction, alcylation and digestion with trypsin.

QUANTITATIVE ANALYSIS

NGAL(Lipocaline-2) is a protein, which could be found in human urine. The normal concentration levels of NGAL in healthy humans are around 10-20 ng/ml. NGAL levels during AKI are heavily increased (200 ng/ml and higher). In a first study it was tested, if a calibration curve of spiked human NGAL in urine could be used for absolute quantitation using the standard addition approach. In parallel it should be tested which protein purification approach give the best results in regards to reproducibility and protein recovery and also in regards of sample throughput and easy sample handling.

The easiest and fastest sample handling process was available using desalting spin columns. Even though there is no concentration effect using this purification device, the detection of the NGAL peptides was possible down to the lowest spiked standard protein concentration. In the opposite, protein precipitation took far more time than any other purification approach and showed high variability in the overall protein recovery from one experiment to another. Therefore all following experiments were performed using either desalting spin columns or filter devices.

NGAL was spiked in human urine in concentrations of 50, 100, 250 and 500 ng/ml. Figure 2 shows the comparison of the calibration curves using desalting spin columns and Amicon filter devices. The reproducibility between three parallel preparations for each concentration was slightly better using Amicon filters than spin columns. But much more important was the fact that for both approaches the calibration curve hit the x-axis instead of the y-axis, which should be expected in the case of a standard addition experiment. This could only be explained by a significant loss of NGAL during the sample preparation, which has an higher influence on the lower concentration than on the higher concentrations and therefore led to a wrong slope of the calibration curve

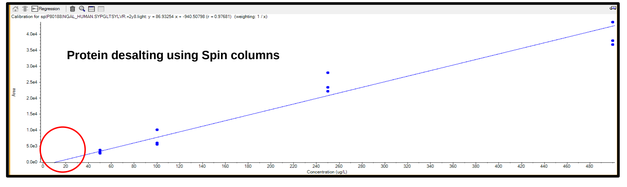


Figure 2: Calibration Curves for peptide SYNVTSVLFR y8
The calibration curves for NGAL peptide SYNVTSVLFR y8 showns as an example the influence of protein loss during sample preparation. This effect could be seen using filter devices as well as spin columns.

Protein losses by unspecific binding on surfaces could be minimized by addition of a high concentrated matrix protein. To find out the necessary amount, human serum albumin was spiked in urine with a fixed NGAL concentration.

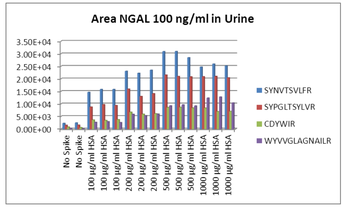
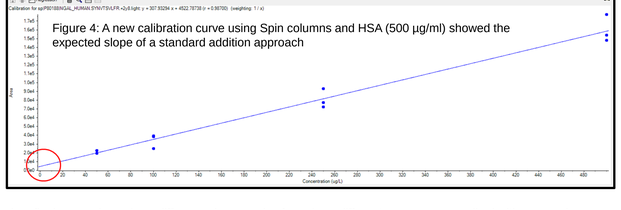


Figure 3: Comparison of NGAL peptide peak areas from three different preparations from urine, spiked with equal amout of NGAL (100 ng/ml), with increasing concentration of human serum albumin (HSA).

The effect of the addition HSA for the recovery of NGAL was extremly high. For most NGAL peptides a concentration of 500 µg/ml HSA gave an 10 fold increase of NGAL peptide peak areas.



In a final comparison, three different urine samples from three different persons were spiked with an equal amount of NGAL. One half of the samples were proccess with HSA the other half without HSA.

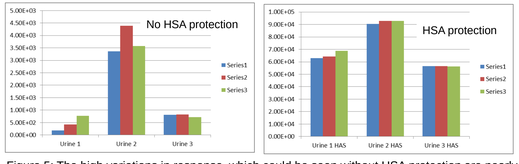


Figure 5: The high variations in response, which could be seen without HSA protection are nearly removed by the addition of HSA. Urine 2, which was much higher concentrated gave higher NGAL signals. Therefore it is necessary in addition to HSA protection to measure the overall protein concentration in urine to have the same concentration in the standards as well as samples.

Comparison MRM vs MRM3

A comparison of results obtained by MRM vs. MRM3 clearly showed the increase of selectivity. Especially for samples, which contains HSA, interferences are much higher and could cause lower reproducibilty. This effect will be even higher in case of faster LC separations.

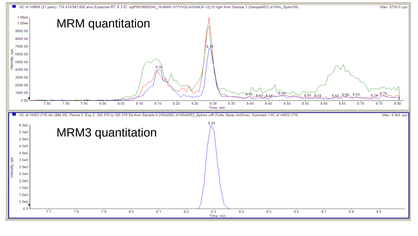


Figure 6: Comparison of the MRM traces for three transitions of the NGAL peptide WYVVGLAGNAILR against the MS3 XICs (MRM3) of the same peptide from the same sample.

CONCLUSIONS

- Amicon filter devices as well as desalting spin columns could be used for sample preparation. Spin column are the fastest way for preparation.
- To avoid protein losses addition of a matrix protein like human serum albumin in a concentration of at least 500 µg/ml is necessary.
- Urine samples should have the same protein concentration as the standard samples → Protein concentration calculation is absolutely necessary.
- Finally a method using a isotopic labeled NGAL standard would be the ultimate method to cover all variability in sample preparation as well as mass spectrometry
- MRM3 offers more selectivity in case of high background.

TRADEMARKS/LICENSING