Development of MRM Assays for Quantification of Low Abundance Biomarkers in Human Serum

Catalin E. Doneanu[1], Weibin Chen[1], Asish B. Chakraborty[1], Johannes P.C. Vissers[2], Johannes M.F.G. Aerts[3], Gordon Fujimoto[1], Michael J. Nold[1], James I. Langridge[2], John C. Gebler[1]

[1]Biopharmaceutical Sciences, Waters Corporation, Milford, MA 01757; [2]MS Technologies Center, Waters Corporation, Manchester, UK; [3]Department of Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

OVERVIEW

- An MRM-based assay for measuring C-reactive protein (CRP), L-selectin, CCL18 chemokine and Chitotriosidase 1 (CHIT1) in human serum, was developed on a Quattro Premier™ XE triple quadrupole mass spectrometer coupled to the nanoACQUITY™ UPLC system. Two biomarkers (CCL18 and CHIT1) were also measured in sera from patients with Gaucher disease.
- C-reactive protein, a diagnostic marker for rheumatoid arthritis [1,2], known to be present in human serum at a median concentration of 0.8 µg/mL [3] was quantified using an external standard calibration method and using an internal standard ^{13}C labeled peptide.
- Coupled with immuno-affinity depletion, the MRM assay can measure serum proteins down to 25 ng/mL.

INTRODUCTION

Human serum is a rich medium for the discovery of prospective biomarkers. It is believed that serum contains tens of thousands of proteins spanning ten to eleven orders of magnitude in protein abundance [4]. However, 99% of the serum protein mass can be attributed to only 22 different high and medium abundance proteins.

The MRM absolute quantification of proteins is based on the assumption that a particular tryptic peptide is a stoichiometric representative of the protein from which is cleaved. Using this method, proteins are normally quantified by either spiking-in a known amount of synthetic stable isotope-labeled peptides as internal standards or using an external calibration plot established from a prior analysis on a set of known concentrations of a tryptically digested protein added in the biological fluid digest.

In this study, two low-abundance serum proteins (CRP and L-selectin), known to be present in human serum at a concentration level of around 1 µg/mL [2,5] were quantified simultaneously by MRM using ^{13}C-isotopically labeled internal standards. CRP concentration was also measured using a calibration plot obtained for the CRP digest spiked in an undepleted human serum digest. In addition, two recently discovered biomarkers, CCL18 chemokine and Chitotriosidase 1 [6,7] were quantified using ^{13}C-labeled peptides in sera obtained from pre-treated and post-treated patients suffering from Gaucher disease, a genetic disorder caused by the deficient activity of the enzyme *glucocerobrosidase*. CCL18/CHIT1 can be effectively used as early predictors for treatment efficacy.

MRM transitions were optimized for each biomarker, using the best responding peptides in electrospray ionization. Other parameters responsible for MS/MS fragmentation, like collision energy and cone voltage were further optimized to enhance the signal intensity for each MRM chromatogram.

Serum samples were processed to enrich low-abundance proteins through the selective removal of Top6/Top12/Top20 most abundant proteins using commercially available immuno-depletion kits.

EXPERIMENTAL PROTOCOLS

1. A 20 µL aliquot of human serum (Gemini Bio, Woodland, CA) was depleted of the six most abundant proteins – Top6 (albumin, IgG, anti-trypsin, IgA, transferrin and haptoglobulin) according to manufacturer's specifications (Agilent, Palo Alto, CA). Another serum aliquot (80 µL) was depleted of twenty high abundance proteins – Top20 (albumin, IgGs, transferrin, anti-trypsin, fibrinogen, haptoglobin, acid-1 glycoprotein, plasminogen, α-2-macroglobulin, ceruloplasmin, apolipoproteins A1, A2, B and complement C1, C3 and C4) using a ProteoPrep immuno-depletion cartridge (Sigma-Aldrich, St. Louis, MO). A 30 µL serum aliquot obtained from Gaucher disease patients was depleted of the top twelve most abundant proteins — Top12 (albumin, IgGs, transferrin, anti-trypsin, fibrinogen, haptoglobin, acid-1 glycoprotein, α-2-macroglobulin, apolipoproteins A1 and A2) using a ProteomeLab IgY-12 LC column (Beckman Coulter, Fullerton, CA).
2. Serum samples were denatured in 0.1% RapiGest™(Waters, Milford, MA), reduced with 5 mM DTT, alkylated with 10 mM IA and digested with sequencing grade trypsin (Promega, Madison, WI) overnight.
3. Recombinant L-selectin (Randox, Crumlin, UK), CCL18 chemokine (PeproTech, Rocky Hill, NJ) and Chitotriosidase 1 (provided by the laboratory of Professor Aerts) were digested with trypsin using the protocol mentioned above. Recombinant C-reactive protein (US Biological, Swampscott, MA) was also digested using the same protocol and spiked in an undepleted human serum digest at the concentration of 0.05, 0.2, 1, 2, 4, 10, 20 and 100 nM to build up an external calibration plot.
4. Two synthetic peptides containing a ^{13}C-labeled leucine, representing the sequence of T5 peptide from CRP (ESDTSYVSLK) and the sequence of T10 peptide from L-selectin (AEIEYLEK) were obtained from New England Peptide (Gardner, MA). Peptides were spiked at a concentration level of 0.33 nM in the Top6 depleted digest and at 1 nM in the Top20 depleted serum digest. Two ^{13}C-isotopically labeled peptides corresponding to T26 (SFTLASSSDTR) and T38 (YPLIQTLR) peptides from CHIT1 were spiked at a concentration of 5 nM in Top12 depleted serum digests obtained from Gaucher disease patients.

Mass Spectrometry and UPLC System

MRM analyses were performed on a Waters Quattro Premier™ XE triple quadrupole mass spectrometer coupled with the nanoACQUITY™ UPLC system (Waters). Five microliters of peptide samples were injected, desalted on a 180 µm x 20 mm Symmetry 5 µm C_{18} trap column and separated on a 75 µm x 100 mm BEH130 C_{18} column packed with 1.7 µm particles, at a flow rate of 300 nL/min.

The gradient consisted of 1-50% mobile phase B over 30 min, followed by 50% B for 10 min (mobile phase A: water with 0.1% formic acid; mobile phase B: acetonitrile with 0.1% formic acid). The following Quattro Premier™ XE parameters were used: electrospray potential 2.8 kV, cone voltage 35/40 V, source temperature 90°C, MS1/MS2 mass window 1 Da, collision energy 15/20 eV and 25 ms dwell time.

RESULTS

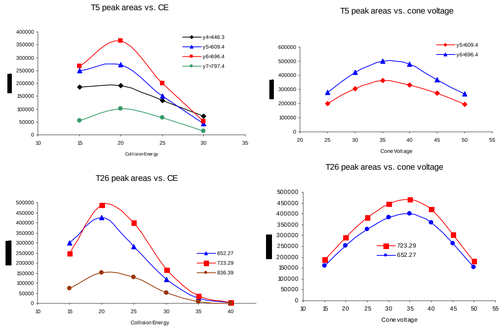

Figure 1. Optimization of collision energy (CE) and cone voltage (CV) for T5 peptide from CRP and for T26 peptide from CHIT1.

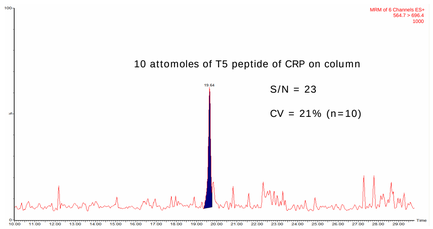

Figure 2. Detection limit for the CRP digest spiked in 1 µM Glu Fib.

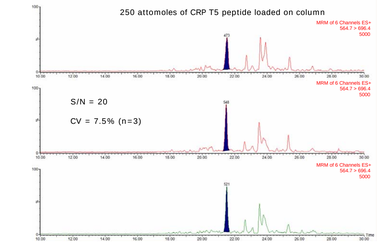

Figure 3. Detection limit for the CRP digest spiked in an *undepleted* human serum digest. Peak areas for three consecutive injections are shown for each MRM trace.

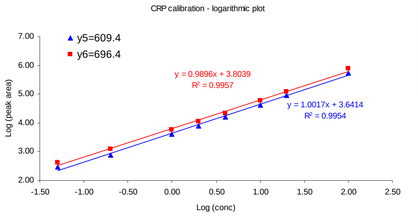

Figure 4. Logarithmic calibration plots obtained for two T5 transitions (y_5 and y_6) for the CRP concentration range of 0.05 to 100 nM.

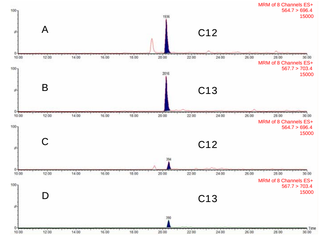

Figure 5. MRM chromatograms of Top20 (A,B) and Top6 (C,D) depleted human serum spiked with 1 nM ^{13}C labeled T5 CRP peptide (Top20) and respectively 0.33 nM ^{13}C T5 (Top6). Peak areas of T5 peaks are shown in each MRM trace.

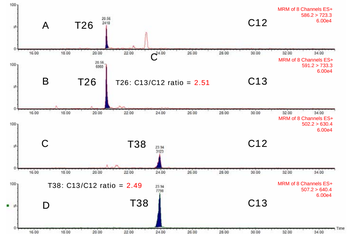

Figure 6. MRM chromatograms of Top12 depleted human serum digest spiked with 5 nM ^{13}C labeled T26 (A,B) and ^{13}C labeled T38 (C,D) peptides from Chitotriosidase 1. The serum sample was obtained from a Gaucher disease patient. Peak areas of T26/T38 peaks are shown in each MRM trace.

	Top6 depleted		Top20 depleted	
Quantification method	C13 IS	CRP calib	C13 IS	CRP calib
Total serum volume (uL)	20	20	80	80
Volume of human serum used for one measurement	44 nL		156 nL	
m/z 564.7 --> 609.4 transition	1.09 ± 0.10	0.82 ± 0.04	0.72 ± 0.02	0.66 ± 0.03
m/z 564.7 --> 696.4 transition	1.06 ± 0.02	0.72 ± 0.04	0.76 ± 0.02	0.63 ± 0.03
Average CRP conc (ug/mL)	1.07 ± 0.10	0.77 ± 0.04	0.74 ± 0.02	0.65 ± 0.03

Table I. Comparison of the CRP levels measured using the external calibration method and the ^{13}C internal standard method.

	Top6 depleted	Top20 depleted
Quantification method	C13 IS	C13 IS
Total serum volume (uL)	20	80
Volume of human serum used for one measurement	44 nL	156 nL
m/z 497.8 --> 681.4 transition	0.76 ± 0.06	1.19 ± 0.12
m/z 497.8 --> 794.4 transition	0.83 ± 0.03	1.11 ± 0.05
Average L-selectin conc (ug/mL)	0.79 ± 0.06	1.15 ± 0.12

Table II. L-selectin serum concentrations measured using the ^{13}C internal standard method in Top6 and Top20 depleted samples.

	Chitiriosidase 1 (CHIT1) serum concentration (µ g/mL)				
	Pre-treatment (1991-1994)		Post-treatment (2001-2005)		CHIT1 conc.
Patient	T26	T38	T26	T38	decreased
Patient 1	11.6 ± 0.6	9.1 ± 0.3	1.8 ± 0.2	1.6 ± 0.1	6 fold
Patient 2	4.3 ± 0.1	4.4 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	3 fold
Patient 3	11.3 ± 0.4	9.4 ± 0.4	3.2 ± 0.2	2.3 ± 0.1	4 fold

Table III. CHIT1 serum concentrations measured in Top12 depleted serum samples obtained from Gaucher disease patients. The quantification is based on using two ^{13}C isotopically labeled peptides.

CONCLUSIONS

- Peptide response was linear over three orders of magnitude for the CRP digest spiked in *undepleted* human serum.
- Excellent sensitivity was achieved for CRP detection, with the ability to detect 10 attomoles of CRP digest loaded onto a 75 µm id column.
- Similar results were obtained for CRP when absolute protein quantification was performed either by using isotopically labeled internal standards or by using the external standard calibration curve.
- CRP and L-selectin were not detected in undepleted serum, but they were detected in Top6/Top20 depleted samples. The limit of quantification for CRP in human serum was 25 ng/mL.
- CCL18 was not detected in Top12 undepleted samples from Gaucher patients probably because is was bound to albumin [7].
- CHIT 1 levels in the sera of Gaucher disease patients decreased only 3-6 fold even after prolonged treatment (10 years).

REFERENCES

1. Kuhn, E et al *Proteomics*, 2004, 4, p1175.
2. Liao H et al *Arthritis & Rheumatism*, 2004, 50, p3792.
3. Ablij H, Meinders A *Eur J Int Med*, 2002, 13, p412.
4. Anderson L, Anderson N *Moll Cell Proteomics*, 2002, 1.11, p845.
5. Atalar E et al *Int J Cardiol*, 2002, 84, p227.
6. Hollak C, van Weely S, van Oers M, Aerts *J Clin Invest*, 1994, 93, p1288.
7. Boot R et al *Blood*, 2004, 103, p33.

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS