Application Note: 380

Targeted Quantitative Protein Analysis in Human Serum, Using High Resolution Multiple Selected Reaction Monitoring Assays

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Key Words

- TSQ Quantum Ultra™
- Surveyor HPLC™
- Biomarkers
- H-mSRM
- Human serum complex sample
- Proteomics
- Targeted protein quantitation

Introduction

A common endpoint for a biomarker discovery experiment is a list of putative marker proteins. A reasonable next step is to then perform targeted quantitative measurements of these proteins in an expanded patient population to assess their validity as markers. Analytical accuracy and precision are required for unambiguous quantitative analysis of targeted proteins from very complex mixtures. Wide dynamic range and high sensitivity are critical for detecting low abundance proteins. Such an assay is also appropriate for "targeted discovery" experiments where the goal is to quantitate a large number (up to hundreds) of known proteins in a complex sample.

One approach for this strategy is the use of tandem mass spectrometry to monitor a unique peptide (or peptides) for each protein of interest by a selected reaction monitoring (SRM) assay, or by simultaneous analysis of many peptides by a multiple selected reaction monitoring (mSRM) assay. This approach can be extended further to provide absolute quantitation of targeted proteins by incorporation of appropriate stable isotope-labeled peptides as internal standards.

While mSRM assays are sensitive for targeted peptides, in a complex matrix, such as human serum, analyte selectivity can become a major issue. It is often difficult to differentiate between the targeted peptide signal and matrix background, particularly when quantifying multiple very low abundance proteins. The unique high resolution SRM (h-SRM) capability of the TSQ Quantum Ultra helps to restate this problem and increase assay specificity.

In this presentation, we demonstrate the TSQ Quantum Ultra mass spectrometer's unparalleled capability for highly sensitive and accurate multiple protein quantitation from human serum by using high resolution multiple selected reaction monitoring (h-mSRM). An h-mSRM assay was developed for detecting 53 targeted proteins in human serum by using both unit mass resolution and high resolution (0.2 FWHM) for the Q1 quadrupole. The sensitivity, reproducibility, dynamic range and overall performance advantages of h-mSRM assays were evaluated. Additionally, a specific h-mSRM assay was developed for detecting a known biomarker (IL-6) from human serum.

Goal

Develop a fast, robust method for accurate, quantitative analysis of many targeted proteins in complex mixtures by using high resolution triple quadrupole mass spectrometry on a TSQ Quantum Ultra instrument.

Experimental Conditions

Sample Preparation

Whole human serum and interleukin-6 were used. The serum was diluted 40 times with 6M Guanidine. One milliter of the diluted human serum sample and 10 µg of IL-6 were reduced and S-carboxymethylated, exchanged into 100 mM ammonium bicarbonate buffer and enzymatically digested. The digested mixtures were dried with a SpeedVac device and reconstituted with 200 µL water containing 0.1% TFA.

Peptide Selection and mSRM Transition Selection

Figure 1 shows two basic approaches for peptide selection and mSRM transition design. If the targeted protein is detected in a previous LC/MS/MS experiments, the peptides which a) had been detected repeatedly from these experiments, b) were unique for one single protein, c) contain no Cys, Met or other commonly modified residues and d) have proper mass range (600–2000 MW) were selected for the mSRM assays. Usually, multiple fragment ions for each selected peptide were used to maximize specificity.

If no HPLC/MS/MS data was available for the targeted protein, an SRM predictor tool (P3 Predictor) was used to predict candidate peptides and multiple fragment ions for SRM assay design (Figure 2). P3 Predictor takes amino acid sequences of targeted proteins of interest and performs in silico digestion. Peptides which contain no Cys, Met, His, NxS(T) modification, R-P or K-P, and meet user-defined peptide length criteria will be listed as candidate peptides. A user simply selects one or multiple candidate peptides from the list and P3 Predictor will predict Q1 and Q3 SRM transitions automatically with proper collision energies and add them to an output csv file which the Quantum Ultra instrument can accept directly. In our experiments, for the 53 major serum proteins, 103 SRM transitions (Table 1) were used based on previous work by Anderson et al.[1] Six SRM transitions (560.82/616.38, 560.82/731.41, 560.82/844.49, 663.36/698.42, 663.36/812.46, 663.36/1012.54) were used for interleukin-6 based on the P3 Predictor tool. The collision energies were assigned by using the standard formula of CE=0.034 x *m/z* +3.314.

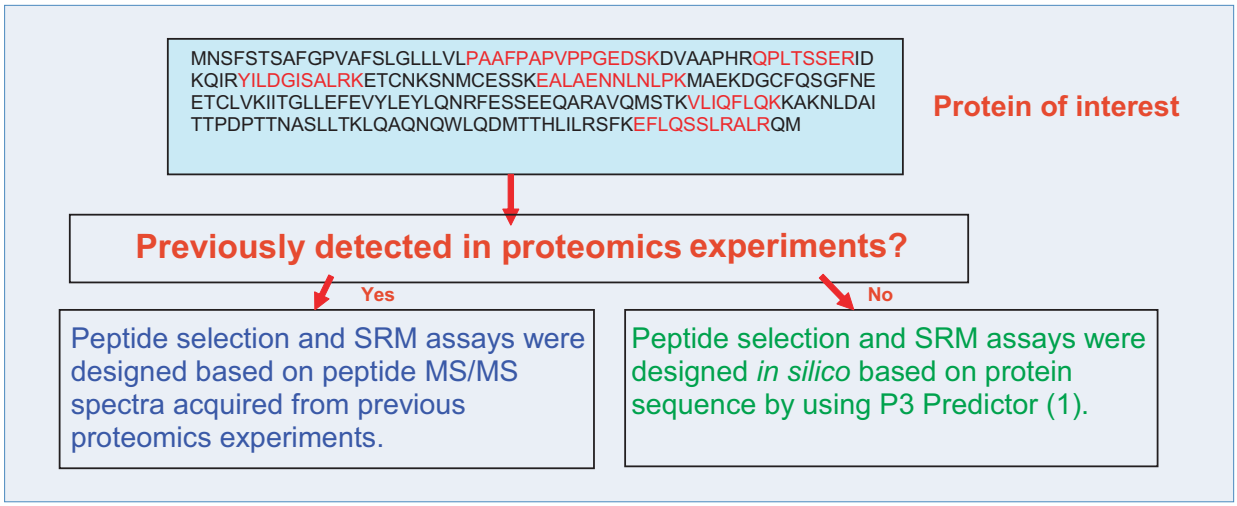

Figure 1. Basic approaches for peptide selection and multiple SRM assay design

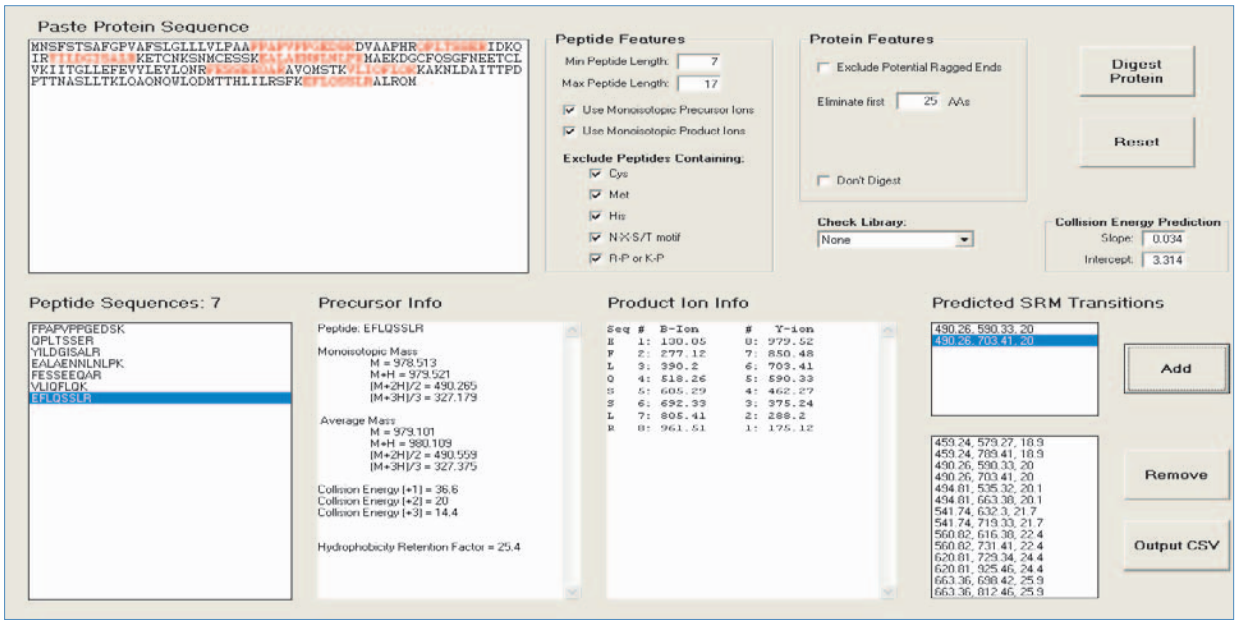

Figure 2: P3 predictor—an multiple SRM transition prediction tool (by Michael J. MacCoss et. al from University of Washington)

LC Separation and MS Analysis

HPLC

A PicoFrit™ C18 column (75 µm×100 mm) was used for peptide separation. A Surveyor™ MS Pump was used to produce and deliver a solvent gradient (A:0.1%FA/2%ACN/98%H_2O, B:0.1%FA/100%ACN) to the column by means of a flow splitter. The post splitter flowrate was 300 nL/min. The linear ramp was from 2% B to 50% B in 85 min. Samples were loaded directly onto the column by a Micro AS autosampler after the flow splitter. The sample loading rate was 5 µL/min and loading time was 15 min.

MS

TSQ Quantum Ultra with Ion Max™ source equipped with a nanoflow column adapter (New Objectives) was used.

For SRM set up:
SRM set up 1: Q1, 0.7 FWHM; Q3, 0.7 FWHM
SRM set up 2: Q1, 0.4 FWHM; Q3, 0.7 FWHM
SRM set up 3: (h_SRM): Q1, 0.2 FWHM; Q3, 0.7 FWHM
Q2: 1.5 mTorr (Ar)
Scan width: 0.002 *m/z*
Scan time: 20ms and 2 ms

For SRM-triggered MS/MS set up:
Scan Event 1
Q1 and Q3: 0.7 FWHM; Q2: 1.5 mTorr;
Scan width: 0.002 *m/z*; Scan time: 20 ms

Scan Event 2
DD precursor mass from Scan Event 1; Q1, 0.7 FWHM; signal threshold 30,000 counts
Q2: 1.5 mTorr, CE: 0.034 × precursor mass *m/z* +3.134;
Dynamic Exclusion™ settings: repeat, 1; duration, 30s; exclusion time, 30s; exclusion list size, 50.

A	B	C	D	E	F	G	H	I	J	K	L	M
Protein	Peptide Sequence	Q1	Q3	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Ave	Std	CV (%)
						Area of total fragment ions						
Afamin	DADPDTFFAK	563.8	825.4	460361	343876	463639	375549	368701	449877	407001	53231	13.1
		563.8	940.4									
Alpha-1-acid glycoprotein 1	NWGLSVYADKPETTK	570.3	575.3	3363991	3149662	4087824	3888870	3181300	3630443	3560348	385063	10.8
		570.3	1052.5									
Alpha-1-antichymortrypsin	EIGELYLPK	531.3	633.4	3570363	3212687	3963132	3359817	3064500	3544359	3452476	316139	9.2
		531.3	819.5									
Alpha-1B- glycoprotein	LETPDFQLFK	619.4	995.5	3815348	3411667	4013810	3945637	3965385	3791761	3823935	219975	5.8
		619.4	894.5									
Alpha-2-antiplasmin	LGNQEPGGQTALK	656.8	771.4	1063920	804714	1221469	1097863	812108	1013693	1002295	165111	16.5
		656.8	900.5									
Alpha-1-antitrypsin	DTEEEDFHVDQVTTVK	631.3	790.4	2103352	2725101	1671968	1594758	1806790	182299	1680711	841187	50
		631.3	889.5									
Alpha-2- macroglobulin	LLIYAVLPTGDVIGDSAK	923.0	1172.6	9116043	7565135	6513412	9040774	8044853	7233501	7918786	1027185	13
		923.0	1059.5									
Angiotensinogen	PKDPTFIPAPIQAK	508.3	556.4	157986	222990	226592	226163	163494	209165	201065	31923	15.9
		508.3	724.4									
Antithrombin-III	DDLYVSDAFHK	437.2	803.4	208027	274147	172331	143431	167764	147782	185580	49084	26.4
		437.2	704.3									
Apolipoprotein A-I	ATEHLSTLSEK	405.9	664.4	8310093	7404901	8768816	7389413	8732920	8181190	8129556	611263	7.5
		405.9	777.5									
Apolipoprotein A-II precursor	SPELQAEAK	486.8	546.4	14682243	13161798	16449486	15604447	15516303	15091455	15084289	1111632	7.4
		486.8	659.4									
Apolipoprotein A-IV	SLAPYAQDTQEK	675.8	982.4	882356	1040338	943183	1086796	1066425	978521	999603	78869	7.9
		675.8	1079.5									
Apolipoprotein B-100	FPEVDVLTK	524.3	803.5	815385	751581	822726	844726	590265	666462	748524	101015	13.5
		524.3	674.4									
Apolipoprotein C_I liprotein	TPDVSSALDK	516.8	719.3	3776528	3585466	4138996	3724512	3760057	4077021	3841930	217222	5.7
		516.8	620.3									
Apolipoprotein C_II liprotein	STAAM*STYTGIFTDQVLSVLK	750.4	1149.7	6787	13319	47439	20992	46405	37616	28760	17437	60.6
		750.4	1002.6									
Apolipoprotein C_III liprotein	DALSSVQESQVAQQAR	858.9	1144.6	1508129	1271980	1643745	1482611	1323109	1294646	1420737	147600	10.4
Apolipoprotein E	LGPLVEQGR	484.8	686.3	1219946	1091977	1272193	1028347	1144104	1103460	1143338	89358	7.8
		484.8	701.4									
Beta-2- glycoprotein I	ATVVYQGER	511.8	751.4	9889076	8814814	10796000	9447598	10202994	10095120	9874267	679873	6.9
		511.8	652.3									
C4b-binding protein alpha chain	LSLEIEQLELQR	735.9	916.5	1350409	1340156	963933	1776467	1643076	1614620	1447944	292755	20.2
Ceruloplasmin	EYTDASFTNR	602.3	695.3	1856971	1680288	1787627	2014691	2167404	1910802	1901281	168670	8.9
		602.3	624.3									
Clusterin	LFDSDPITVTVPVEVSR	937.5	1296.7	2395112	2520417	2382234	2690174	2635690	2777964	2566915	161392	6.3
		937.5	686.4									
Coagulation factor V	DPPSDLLLLK	555.8	898.6	2957516	3484642	4611308	2649725	3077363	3572073	3392105	687963	20.3
Coagulation factor XIIa heavy chain	VVGGLVALR	442.3	784.5	668570	618838	826962	698422	898715	692279	733964	106135	14.5
		442.3	685.4									
Complement C3	TGLQEVEVK	501.8	731.4	4566881	4737491	5720918	5120795	5411222	5561088	5186399	461795	8.9
		501.8	603.3									
Complement C4 gamma chain	ITQVLHFTK	362.9	645.4	111095	112564	100589	159819	133237	128646	124325	21141	17
		362.9	744.4									
Complement C4 beta chain	VGDTLNLNLR	557.8	843.5	2302932	2038312	1821734	2460076	1632647	2100302	2059334	303503	14.7
		557.8	629.4									
Complement factor C9	AIEDYINEFSVR	728.5	1271.6	185702	212040	174997	161374	230387	205447	194991	25570	13.1
		728.5	1027.5									
Complement factor B	EELLPAQDIK	578.4	671.4	2011175	2312117	2128099	2011175	2447949	2128099	2173102	173987	8
		578.4	784.5									
Complement factor H	SPDVINGSPISQK	671.4	830.4	330940	425920	267351	267545	427823	267351	331155	78120	23.6
		671.4	572.3									
Fibrinogen alpha chain	GSESGIFTNTK	570.8	780.4	110715	94678	137387	116792	138690	138164	122738	18299	14.9
		570.8	867.5									
Fibrinogen beta chain	QGFGNVATNTDGK	654.8	706.3	64139	61523	76351	87158	91996	88959	78354	13156	16.8
		654.8	805.4									
Fibrinogen gamma chain	DTVQIHDITGK	409.5	670.4	980969	1127408	1211679	1290416	1105767	1122725	1139811	104664	9.2
		409.5	533.3									
Fibronectin	DLQFVEVTDVK	647.3	789.4	148997	79771	95323	118285	113737	124585	113433	23945	21.1
		647.3	690.4									
Gelsolin, isoform 1	TGAQELLR	444.3	786.5	422213	430807	504372	465432	559462	569154	491907	63210	12.9
		444.3	729.4									
Haptoglobin beta chain	VGYVSGWGR	490.8	562.3	36340479	38692289	43475501	35250502	44157166	46000993	40652822	4482765	11
		490.8	661.3									
Hemopexin	NFPSPVDAAFR	610.8	959.6	19718474	17178303	21208304	18586954	17250682	21933002	19312620	1997910	10.3
		610.8	775.3									
Heparin cofactor II	TLEAQLTPR	514.8	814.4	1176390	1501771	1764117	1165217	1594067	1725999	1487927	262912	17.7
		514.8	685.4									
Histidine-rich glycoprotein	DSPVLIDFFEDTER	841.9	1171.5	1249897	1304182	1606963	1714889	1660153	1811153	1557873	228422	14.7
		841.9	1058.4									
Inter-alpha-trypsin inhibitor heavy c	AAISGENAGLVR	579.4	629.4	2118520	2104894	2392367	2244207	2302923	2246588	2234748	109605	4.9
		579.4	902.5									
Inter-alpha-trypsin inhibitor light	AFIQLWAFDAVK	704.9	836.4	1316403	1797675	1834854	1347062	1395663	1588799	1546743	229564	14.8
		704.9	949.5									
Kininogen	TVGSDTFYSFK	626.3	1051.4	2601441	2998187	3752582	2872619	2383236	3727717	3055964	571426	18.7
		626.3	994.5									
L-selectin	AEIEYLEK	497.8	794.4	77482	105168	91786	96749	102618	97221	95171	9864	10.4
		497.8	681.3									
Plasma retinol-binding protein prec	YWGVASFLQK	599.8	849.5	2178485	1964456	2480949	2284090	2047641	2444580	2233367	208950	9.4
Plasminogen	LFLEPTR	438.3	615.4	2221967	1958030	2432750	2097034	1719680	2008048	2072918	242677	11.7
		438.3	502.3									
Prothrombin	ETAASLLQAGYK	626.3	879.5	1543698	1348273	1748347	1559187	1602953	1570716	1562196	128452	8.2
		626.3	679.4									
Serum albumin	LVNEVTEFAK	575.4	937.4	580114007	506210531	661792006	512338571	503622280	583128349	5.58E+08	62637353	11.2
		575.4	694.4									
Serum amyloid P-component	VGEYSLYIGR	578.8	1057.5	605914	504502	508405	563431	552861	554938	548342	37744	6.9
		578.8	871.5									
Transferrin	EDPQTFYYAVAVVK	815.4	1160.6	8844379	7043347	9137074	8080055	8450470	8322479	8312967	727562	8.8
		815.4	1288.7									
Transthyretin	AADDTWEPFASGK	697.8	921.4	6443763	6682339	7274622	6840174	5414265	7032782	6614666	653601	9.9
		697.8	606.4									
Vitamin D-binding protein	THLPEVFLSK	585.8	819.5	3237009	3218221	4293045	3497041	3206544	3713418	3527546	425348	12.1
		585.8	932.5									
Vitamin K-dependent protein C	WELDLDIK	516.3	716.4	168787	145466	258455	158618	205679	200416	189570	41128	21.7
		516.3	603.3									
Vitronectin	FEDGVLDPDYPR	711.9	875.4	1924890	1666834	1919119	1972180	1809208	2005032	1882877	124962	6.6
		711.9	1031.5									
Zinc-alpha-2-glycoprotein	EIPAWVPFDPAAQITK	891.9	1087.7	2062315	2009293	2619535	2236890	1768158	2666391	2225430	358207	16.1
		891.9	728.4									

Table 1. Multiple SRM assays and reproducibility for 53 major serum proteins with TSQ Quantum Ultra

Results

Quantitative results for 53 targeted major serum proteins

A total of 103 unique SRM transitions were chosen as proteotypic peptides for the 53 targeted proteins of interest. Using identical human serum samples, five nanoflow HPLC-MS/MS experiments were performed for each sample, with the same 103 SRMs monitored with different resolutions (0.2, 0.4 and 0.7 FWHM) and Q2 dwell times (2 and 20 ms). The high resolution SRM assays (Q1: 0.2 FWHM) gave the best results and clearly resolved targeted analyte transitions from interference peaks that were seen at lower Q1 resolutions. Figure 3 shows one example where high resolution was used to unambiguously detect peptide QGFGNVATNTDGK (representing fibrinogen beta chain). Importantly, it should be noted that 0.2 FWHM data revealed the presence of significant matrix interference in 25% of the SRM transitions which were monitored. The 103 SRMs were detected with enough scans for reliable quantitation using both 2 ms and 20 ms scan times, although the 2 ms scan time gave twice as many scans with only a minor decrease in signal intensity (Figure 4). For narrow peak widths, such as those associated with uHPLC, 2 ms scan times will be required.

For testing this method's reproducibility, the same h-mSRM experiment at Q1: 0.7 FWHM and 20 ms scan time was repeated six times and the results were summarized in Table 1. Among the 53 targeted proteins,

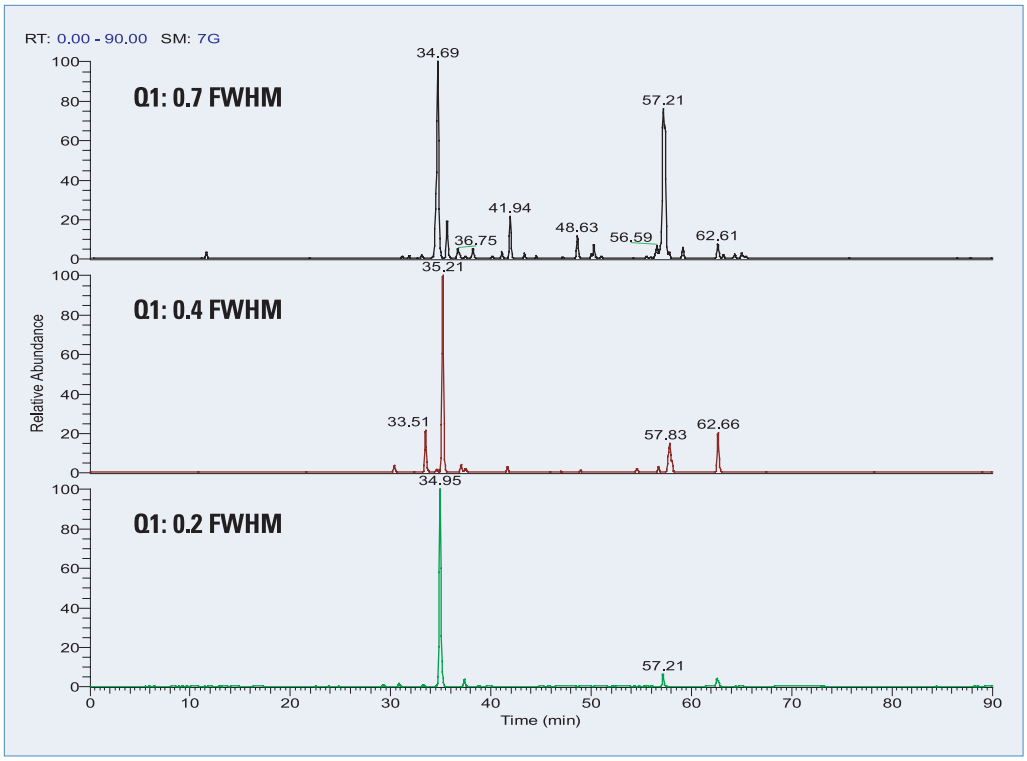

Figure 3. SRM traces for peptide QGFGNVATNTDGK (fibrinogen beta chain) at different Q1 resolution settings, showing the power of high resolution SRM (h-SRM) for unambiguious detection of targeted peptides from human serum. Note the marked specificity of the Q1 0.2 FWHM trace relative to those at 0.4 and 0.7. This performance is achieved with very little reduction in signal intensity.

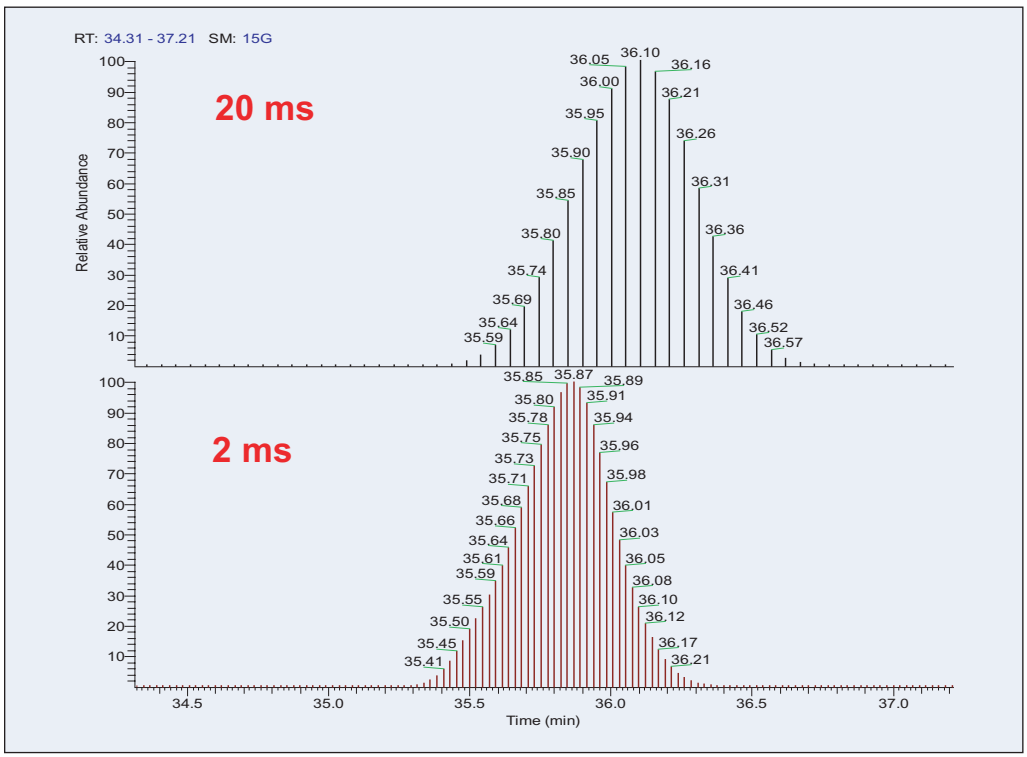

Figure 4: SRM traces for peptide LGPLVEQGR with different SRM dwell times

51 proteins produced acceptable quantitative data, while only two were not reliably observed. For the whole serum digests, CVs (n=6) were from 5–26% (50% of SRMs had CVs 10%). Proteins present at concentrations down to µg/mL levels, such as L-selectin and fibronectin,[1] were reliably detected, yielding a dynamic range of greater than four orders of magnitude (from lowest peak areas of 6E+04 from fibrinogen beta chain to the highest peak areas of 6E+08 from albumin peptide) in a single experiment. MS/MS spectra were acquired once the SRM intensity exceeded 30,000 counts and typically of good quality and showed rich y series and some b series fragment ions, permitting database searching with SEQUEST® in BioWorks™ 3.3 (Figure 5).

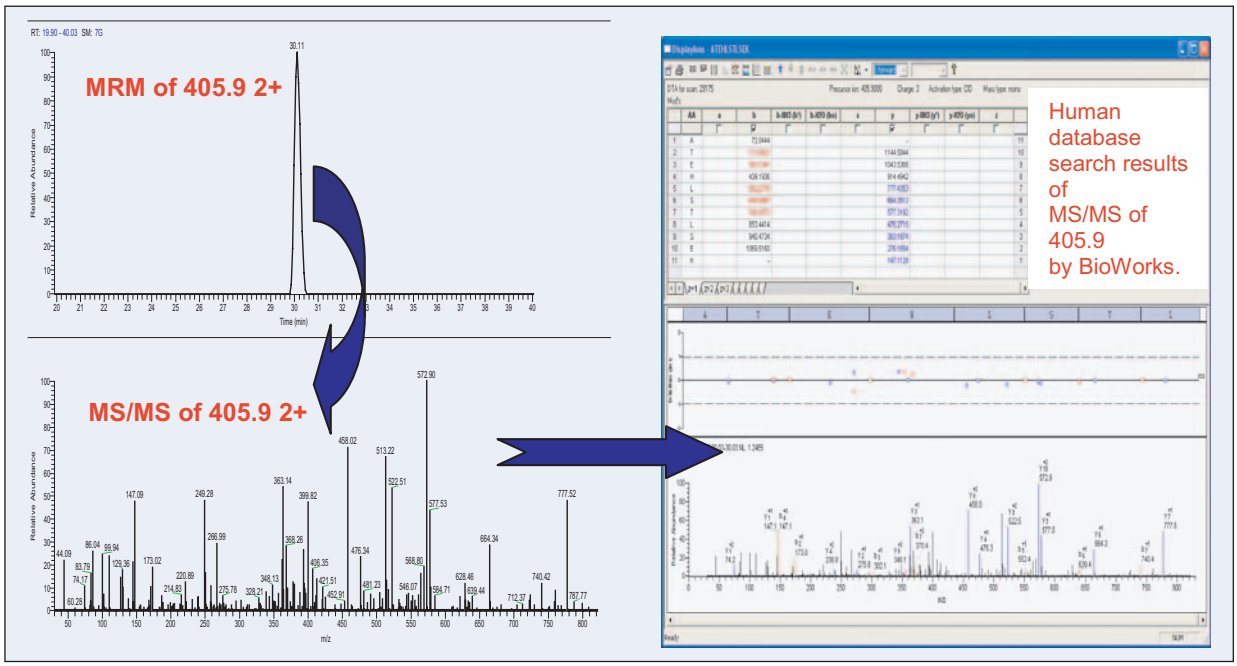

Figure 5: SRM transition for peptide ATEHSTLSEK (Apolipoprotein A-I) triggered the MS/MS spectrum shown below. Database searching with SEQUEST in BioWorks confirms that the peptide structure agrees with the predicted transition.

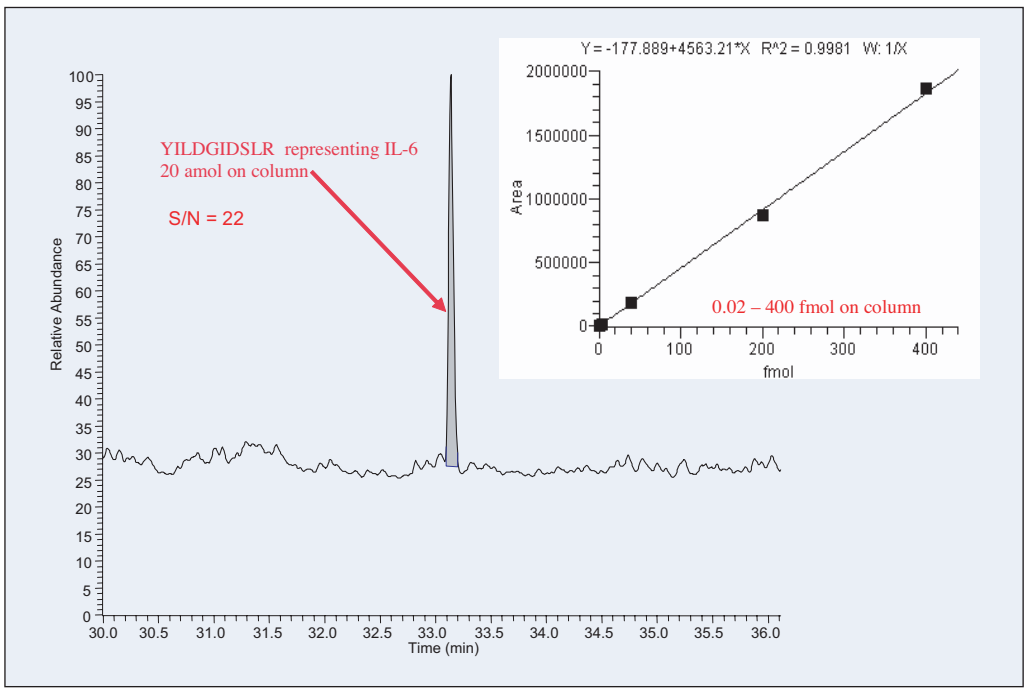

Figure 6: Detection limit and linear dynamic range of IL-6 spiked into human serum

Detection limit and linear dynamic range of IL-6, using h-mSRM assays developed with P3 Predictor

An IL-6 digest mixture was spiked into human serum to create a dilution series, and was run sequentially to evaluate the detection limit and linearity of the h-mSRM assay. Excellent analytical sensitivity and linearity were seen (Figure 6). The linear dynamic range for spiked IL-6 was over four orders of magnitude (0.02–400 fmol on column) and the limit of detection was 20 amol on column with a S/N of 22.

Conclusions

An h-mSRM- based assay for targeted proteins in human serum was developed on a TSQ Quantum Ultra triple quadrupole mass spectrometer. A total of 103 SRM transitions were monitored for the quantitation of 53 serum proteins. One exogenous protein, IL-6, was spiked to evaluate assay performance. Several unique features of the Quantum Ultra instrument contributed to the specificity, sensitivity, and robustness of this assay.

1) Q1 resolution of 0.2 FWHM dramatically reduced non-specific matrix interference from the serum background, dramatically improving assay specificity. At Q1 resolution of 0.4 or 0.7 FWHM, significant interference was seen in 25% of targeted transitions.

2) Analytical assay performance was excellent.

a) %CV varied from 5-26%, with 50% of protein CVs < 10%.

b) Peptide response was linear over four orders of magnitude.

c) Sensitivity was excellent, with the ability to detect proteins present at µg/mL levels and to detect IL-6 at levels as low as 20 amol on column.

d) SRM-triggered MS/MS spectra were of good quality and in most cases sufficient to permit confirmation of peptide ID by database searching with SEQUEST.

References

(1) Leigh Anderson and Christie L. Hunter (2006) Quantitative Mass Spectrometric Multiple Reaction Monitoring Assays for Major Plasma Proteins. Mol. Cell. Proteomics 5.4, 573-588.

(2) Michael J. MacCoss et. al. (2006) Private communication.

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