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Application News

SSI-LCMS-017

Liquid Chromatography Mass Spectrometry

Analysis of Human IgG using Automated Digestion Coupled Directly to LCMS-8050

Perfinity-LCMS-8050

Summary: Quantification of human IgG using an automated online trypsin digestion platform coupled directly to LCMS-8050 triple quadrupole mass spectrometer was performed. Two common peptides for IgG were monitored and the limits of detection were in the low fmol levels on column.

Background: Immunoglobulin G (IgG) is an antibody isotype that is the most abundant antibody found in human serum and is one of the major components of the immune system. Many pharmaceutical companies and research laboratories are interested in studying IgG for its therapeutic and clinical advances in today's market.

Method: Human IgG and bovine serum albumin (BSA) were obtained from Sigma Aldrich in St. Louis, MO. Approximately 250 mg of BSA was weighed and diluted in 50 mL of TRIS buffered saline to yield a 0.5% (w/v) solution. Varying amounts (3 mg, 1.7 mg, 0.75 mg, 0.46 mg, 0.17 mg) of IgG were weighed out and then diluted in 500 µL of the 0.5% BSA solution. Then 200 µL of each IgG standard was added to 300 µL of 6M guanidine in TRIS buffer. Each sample was reduced with dithiothreitol (DTT) at 60°C for 1 hour and then alkylated with iodoacetimide (IAA) in the dark at room temperature for 1 hr. Finally, the sample was quenched with TRIS buffer to give the final concentrations listed in **Table 1**.

Perfinity Workstation-Fraction Collection: Five µL injections of the reduced/alkylated samples were injected onto an Perfinity immobilized enzyme reactor (IMER) trypsin column for automated digestion. Digestion time was 6

Level	Conc. (µg/mL)	Amt. on column (ng)	Amt. on column (fmol)
1	1170	29.25	180
2	663	16.58	100
3	292.5	7.32	46
4	179	4.48	28
5	66	1.65	10

Table 1: Calibration levels for IgG. Concentration levels are those before injected onto Perfinity Workstation and corresponding amounts on column.

minutes at 50°C. The resulting peptides were then trapped onto an online desalting column and eluted at 1 mL/min with a mobile phase gradient from 2-60% B in two minutes. Mobile phase A consisted of 98% water, 2% acetonitrile and 0.1% formic acid. Mobile phase B consisted of 10% water, 90% acetonitrile and 0.1% formic acid. All the peptides were collected into one fraction of approximately 2 mL into a 96 multiwell plate. The multiwell plate was then transferred to the LC/MS/MS for further analysis.

LCMS-8050 Analysis: Scan, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) methods were used for MS analysis. The details of these events are described in **Table 2**. Positive ion heated electrospray ionization (hESI) was used for ionization of IgG peptides collected from the Perfinity workstation. The MS temperature and gas parameters are listed in **Table 3**.

A Waters Acquity C18 (1.7µm x 2.1mm x 100mm) column was used with a binary gradient consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The gradient conditions are shown in **Table 4**. The flow rate was 0.400 mL/min and the column temperature was 50°C. The injection volume was 10 µL.

Perfinity Workstation-LC/MS/MS: For the online coupling of the Perfinity workstation to the LCMS-8050 mass spectrometer, the sample digestion conditions were used as previously described. The peptides were eluted onto a Waters Acquity C18 (1.7µm x 2.1mm x 100mm) column with a binary gradient consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile with the gradient conditions as shown in **Table 4**. The flow rate was 0.400 mL/min and the column temperature was 50°C. The injection volume was 5 µL.

Novilytic Noviplex cards: Mouse whole blood was spiked with a 10 µg/mL standard of R/A IgG and then processed on a Noviplex card. A 50 µL aliquot of spiked whole blood was pipet ted onto the card and allowed to sit for three minutes. After three minutes elapsed, the top layer of the card was peeled back, leaving a membrane of mouse plasma. The membrane was allowed to dry for 10 minutes and then submerged in 27 µL of TRIS buffer. Next, 5 µL of the supernatant was then injected on the Perfinity Workstation coupled directly to the LCMS-8050. An affinity protein G column was used to enrich the IgG from the mouse plasma, and then the sample was digested for 6 minutes at 50°C. The same LC and LCMS conditions were used as described above. A schematic of the Noviplex card is shown in **Figure 1**.

Compound Name	Transitions	+/-	Q1 Rod Bias (V)	CE (V)	Q3 Rod Bias (V)
VVSVLTVLHQDWLNGK	937.70>836.25	+	-27	-28	-26
	937.70>723.95	+	-27	-30	-22
TTPPVLDSDGSFFLYSK	603.70>805.7	+	-22	-16	-13

Table 2: MRM event table for 2 peptides from IgG monoclonal antibody.

MS Parameters	Values
Interface Type	Heated ESI
Nebulizing Gas Flow (L/min)	2
Heating Gas Flow (L/min)	10
Interface Temperature (°C)	300
DL Temperature (°C)	250
Heat Block Temperature (°C)	400
Drying Gas Flow (L/min)	10
CID Gas Pressure (kPa)	350

Table 3: LCMS-8050 MS parameters.

Time (min)	%B
0	2
0.2	2
8	50
9.5	50
10	90
12.5	90
12.51	2
16	2

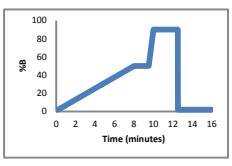

Table 4: LC gradient and graph.

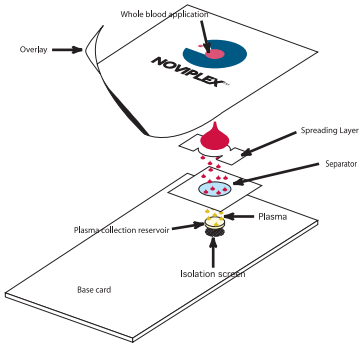

Figure 1: Schematic of Noviplex cards.

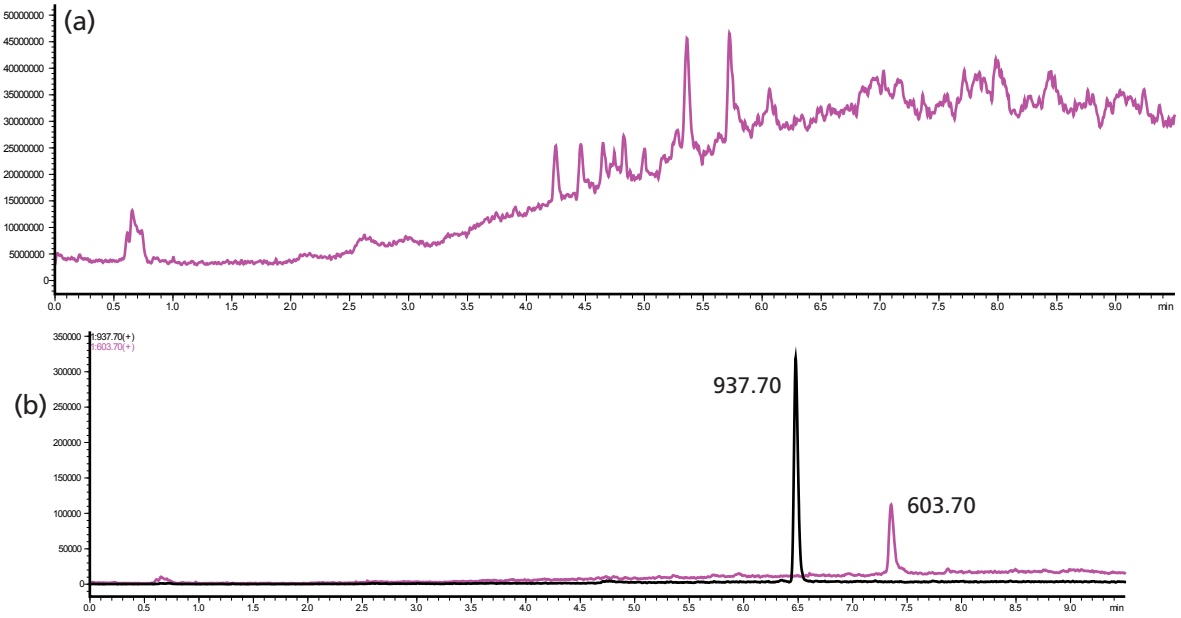

Figure 2: (a) TIC for IgG peptides on LCMS-8050. (b) Selected ion monitoring (SIM) for two peptides (937.70 m/z and 603.70 m/z).

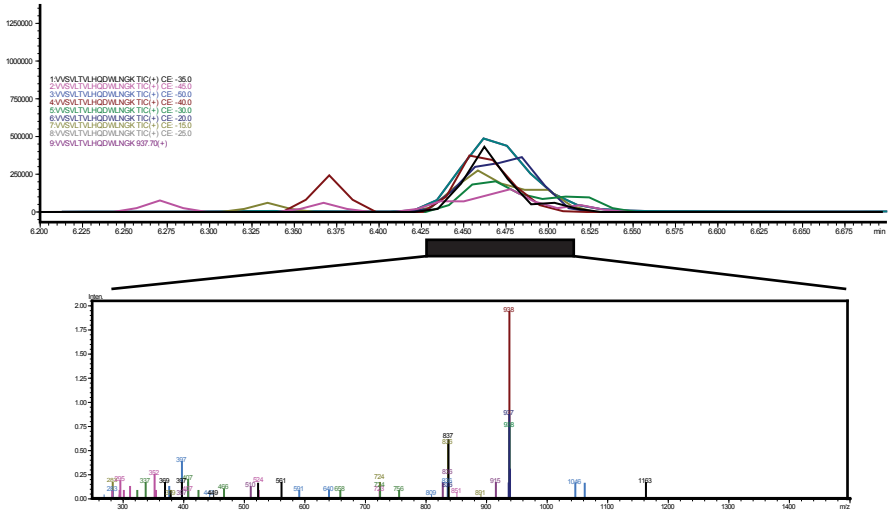

Figure 3: (a) MS product ion scan for precursor 937.70 m/z. CE ranging from -50 to -15 were analyzed. (b) MS spectrum for the product ion scan. Product ions 836.25 and 723.95 were identified.

Results and Discussion: First, the collected fractions were analyzed on the LCMS-8050 using scan and selected ion monitoring (SIM) modes. The SIM m/z values were 937.70 and 603.70 which corresponds to the peptides, VVSVLTVLHQDWLNGK and TTPPVLDSDGSFFLYSK, respectively. The chromatograms for both the scan and SIM modes are shown in **Figure 2**. MRM optimization of the peptides was performed as shown in **Figure 3**. Eight different collision energies were selected to determine which collision energy produced the most intense fragment ions. The MS/MS of the peptide, VVSVLTVLHQDWLNGK, produce two product ions of 836.25 m/z and 723.95 m/z. These products ions were then used for the MRM transitions for this peptide.

The calibration curves were linear in the range tested with r^2 values of 0.994 and 0.996 for the two peptides. Each peptide had a limit of detection (LOD) of 1.65 ng (10 fmol) on column. The calibration curves for the peptides are shown in **Figure 4** and the MS chromatograms for each peptide at levels 1 and 5 are shown in **Figure 5.**

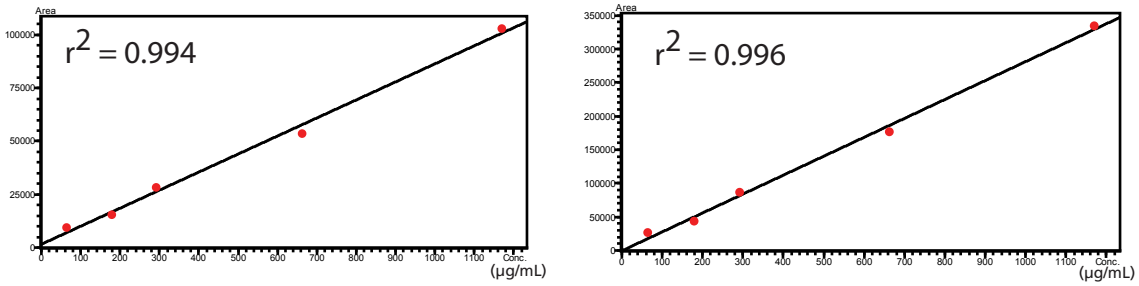

Figure 4: Linear calibration curves for the two peptides from IgG.

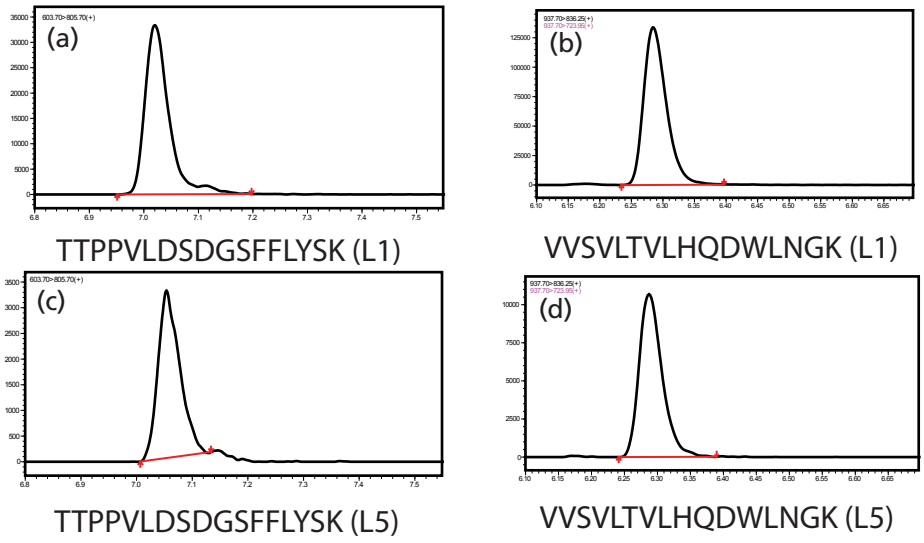

Figure 5: MS chromatograms for the two IgG peptides at the highest level 1 (a-b) and lowest level 5 (c-d).

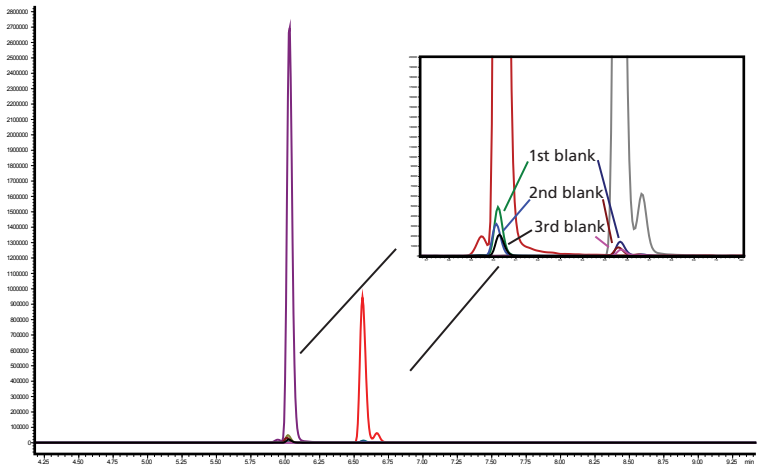

Figure 6: 66 µg/mL IgG standard sample, followed by three blank injections. Inset shows zoomed in region of the three blank injections.

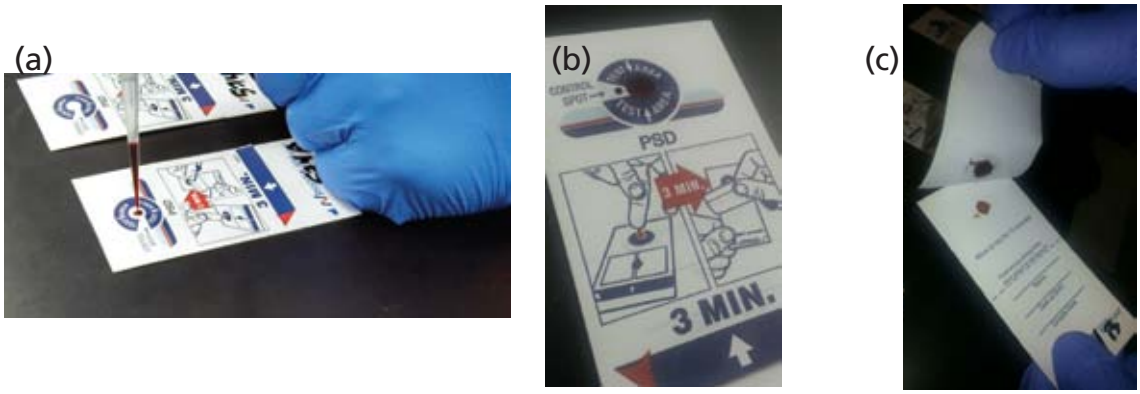

Figure 7: (a) Placement of spiked mouse whole blood onto Noviplex card. (b) Wait three minutes. (c) Peel back top layer to collect plasma from plasma collection disc.

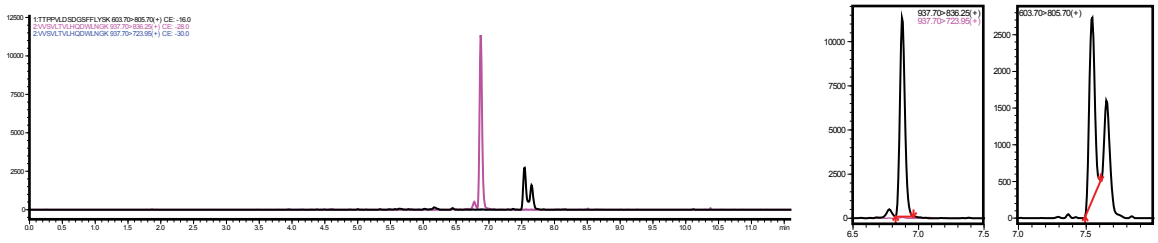

Figure 8: (a) LCMS chromatogram for two IgG peptides extracted from mouse whole blood (b) MS chromatograms for the two IgG peptides.

To assess carryover, three blank injections were performed after direct injection of the 66 µg/mL IgG standard from the Perfinity workstation into the LCMS-8050. For the peptide VVSVLTVLHQDWLNGK, the carryover was 1.7%, 1.2% and 0.5% for the three blanks and for the peptide TTPPVLDSDGSFFLYSK, the carryover was 1.5%, 0.8%, and 0.5%. These values are within specific parameters for cleanup of the reversed phase column after analysis of IgG samples. This is shown in **Figure 6**.

Figure 7 shows the process for using the Novilytic Noviplex cards. The whole blood is placed onto the card and then allowed to activate for three minutes. The membrane holds exactly 2.5 µL of plasma which can then be analyzed using LCMS. Both peptides from IgG were detected using the spiked whole blood and Noviplex cards followed by affinity selection and automated digestion on the Perfinity workstation, as shown in **Figure 8**.

Conclusion: The combination of the Perfinity Workstation coupled to LCMS-8050 was successfully able to quantify IgG into the low fmol range, performing automated online digestion in under 6 minutes. Two common peptides were monitored and quantified using MRM transitions and linear calibration curves. The speed of the digestion coupled to the speed and sensitivity of the LCMS-8050 allows for high throughput and high sensitivity analysis of IgG.

ULTRA FAST MASS SPECTROMETRY

LCMS-8030 LCMS-8040 LCMS-8050 LCMS-2020 LCMS-IT-TOF

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