Advanced Multi-pump Setups for LC-MS Applications in the BioPharma QC Environment

Martin Samonig, Sabrina Patzelt, Carsten Paul, Martin Ruehl, Jenny-Marie T. Wong, and Remco Swart; Thermo Fisher Scientific, Dornierstraße 4-7, 82110 Germering, Germany

ABSTRACT

In the biopharma QC environment, peptide mapping with LC-MS is an analytical tool utilized in lot release of Biotherapeutics. Increasing the throughput of this approach is required by the biopharmaceutical industry. Multi-pump UHPLC systems can be configured to enable tandem analysis with two columns in parallel and thereby decrease the mass spectrometer (MS) idle time. The actual run time is shortened by removing the wash and re-equilibration steps from total run time, which are carried out offline when the second column is running the separation. Another common assay for batch release in biopharma QC labs is LC-MS analysis of intact proteins to assess exact mass or determine impurities. Here, the technique of choice is a desalting step followed by reversed phase LC-MS. The multi-pump system can be easily configured to run also this intact protein workflow.

TANDEM UHPLC OPERATION FOR HIGH THROUGHPUT PEPTIDE MAPPING ANALYSES

INTRODUCTION

Common liquid chromatography (LC) methods with gradient elution can be segmented into an analytical gradient section and a reconditioning section. The gradient section is responsible for the actual chromatographic separation, and the reconditioning section is used for the column wash and re-equilibration for the next injection. The process of column re-equilibration involves replacing the mobile phase between the particles (interparticle), within the pores of the particles (intra-particle), and in the interfacial region between the mobile phase and stationary phase. Good and accepted practice suggests using at least five column volumes to sufficiently equilibrate the analytical column.

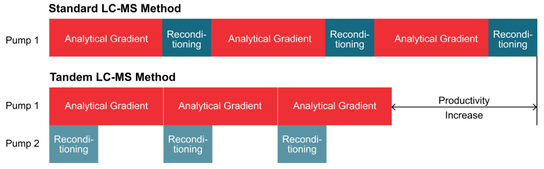


Figure 1. Standard LC-MS method compared to tandem LC-MS method.

Many UHPLC peptide mapping methods require lengthy periods of column washing and equilibration between separations. To possibly increase throughput and mitigate these delays without changing the chromatographic gradient section, a tandem LC approach with a two-pump setup and column switching capabilities can be implemented. In this setup one column is used for the ongoing separation, while the second column is switched offline from the mass spectrometer (MS) and simultaneously washed and conditioned for the next injection (Figure 1).

MATERIALS AND METHODS

50 µL infliximab drug product (Hospira® UK Limited, Leamington Spa, United Kingdom), adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided in the kit. The solution was then transferred to a reaction tube containing 15 µL of the SMART Digest resin slurry, corresponding to 14 µg of heat-stable immobilized trypsin. Tryptic digestion was allowed to proceed at 70° C for 45 min at 1400 rpm. After the digestion, the reaction tube was centrifuged at 7000 rpm for 2 min, the supernatant was transferred to a new tube, and the centrifugation step was repeated. The non-reduced sample was diluted with 0.1% formic acid (FA) in water to a final protein concentration of 100 ng/µL, and 1.0 µg was loaded on the column for all runs.

LC-MS Analysis

The Thermo Scientific™ Vanquish™ Horizon UHPLC system chosen for this setup, consisting of two binary high-pressure gradient pumps (HPG) used as a analytical and reconditioning pump. The lower and upper switching valve of the Vanquish Thermostated Column Compartment (VTCC) was used to switch between the two flow paths and two analytical columns (Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.1 x 250 mm) see figure 2.

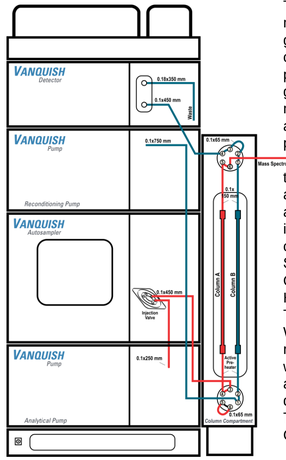
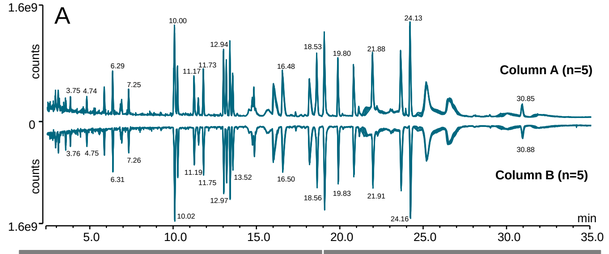


Figure 2. Vanquish UHPLC tandem LC setup with 2-position/6-port (2p6p) valve configurations and required fluidic connections.

The analytical pump was utilized to deliver a 43 min water/acetonitrile + 0.1% formic acid gradient to separate the peptides on one column. Simultaneously the reconditioning pump was delivering a wash and equilibration gradient to recondition the other column for the next injection. At the end of the gradient the analytical pump was set to start conditions to perform a void volume purge and equilibrate the fluidics from the analytical pump to the lower switching valve. At 42 min the lower and upper switching valve toggled the position and the next sample could be immediately injected on the already equilibrated column. The column outlets were coupled to the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole Orbitrap mass spectrometer equipped with a HESI-II probe for mass spectrometric detection. This setup used the Vanquish Variable Wavelength Detector (VVWD) to monitor the reconditioning step, to ensure that no peptides were eluting from the column during this stage, and to confirm proper column equilibration. The data were acquired and processed with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4.



B	Single Column Setup						Tandem LC Setup			
	Column A			Column B					Column A/B	
	RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	RT [min] n=5	RT RSD [%] n=5	Area RSD [%] n=5	Abs. RT Shift Column A to B [min]	Rel. RT Shift Column A to B [%]	RT RSD [%] n=10	Area RSD [%] n=10
	3.75	0.18	2.72	3.76	0.13	1.64	0.005	0.13	0.16	2.12
	4.74	0.054	3.53	4.75	0.11	5.05	0.010	0.21	0.14	4.24
	6.29	0.072	2.33	6.31	0.037	1.14	0.020	0.32	0.18	2.19
	7.25	0.018	4.94	7.26	0.033	4.72	0.016	0.23	0.12	4.94
	10.00	0.032	3.05	10.02	0.037	1.75	0.023	0.23	0.12	2.35
	11.17	0.040	3.96	11.19	0.047	2.70	0.022	0.20	0.11	3.22
	11.73	0.043	1.64	11.75	0.007	2.59	0.025	0.21	0.12	2.15
	12.94	0.014	4.19	12.97	0.012	1.61	0.023	0.18	0.10	3.03
	13.49	0.028	1.66	13.52	0.025	3.11	0.024	0.18	0.10	2.36
	16.48	0.056	1.02	16.50	0.031	0.78	0.024	0.14	0.087	0.91
	18.53	0.019	1.94	18.56	0.020	1.90	0.027	0.15	0.080	2.35
	19.80	0.019	0.50	19.83	0.016	0.78	0.029	0.15	0.078	0.62
	21.88	0.028	4.35	21.91	0.0075	1.71	0.033	0.15	0.083	3.78
	24.13	0.025	1.52	24.16	0.030	0.60	0.031	0.13	0.072	1.09
	30.85	0.039	1.56	30.88	0.039	2.00	0.031	0.10	0.064	1.74
	Average	**0.045**	**2.59**		**0.039**	**2.14**	**0.023**	**0.18**	**0.11**	**2.47**

Figure 3. Reproducible results for the Vanquish tandem LC - Q Exactive HF setup, showing the overlay of five TIC chromatograms for the separation of digested infliximab using the SMART Digest Kit (A) and detailed retention time / area RSD values (B).

RESULTS AND DISCUSSION

Using the tandem LC setup for LC-MS peptide mapping experiments, or more precisely for the separation of the tryptic digested monoclonal antibody infliximab, gave reproducible and confident results as demonstrated in the total ion current (TIC) chromatogram overlay of five replicates (Figure 3A) on two analytical columns with automated alternating column regeneration. Retention time relative standard deviation (RSD) values below 0.11% were achieved for the UHPLC system in tandem column operation compared to 0.045% and 0.039% for the single column setup (Figure 3B). Polar tryptic peptides eluting between 0 and 14 min had the highest RSD values up to 0.18%, and the heavy chain peptide (D151-Y183) at 30.85 min had the lowest with 0.064%. The average absolute retention time shift between column A and column B was 0.023 min (relative, 0.18 %) and shows that peak assignment based on retention time is not impaired. An average peak area RSD value of 2.47% demonstrates the suitability for quantitative data analysis using the tandem LC setup.

A ON-LINE SOLID PHASE EXTRACTION SETUP FOR THE LC-MS ANALYSIS OF THERAPEUTIC PROTEIN MIXTURES

INTRODUCTION

Solid-phase extraction (SPE) is a commonly used laboratory technique to isolate analytes of interest from complex matrices. In the online-SPE approach for the enrichment of low abundant compounds in liquid chromatography, the sample is pre-concentrated on a trap column prior to chromatographic separation. This technique can be applied for the sample cleanup of protein mixtures often containing high amounts of nonvolatile salts, which are present in various biopharma formulation buffers. The presence of such buffers may interfere with the operation of electrospray ion sources by suppressing ionization. In this study, the Vanquish UHPLC setup for fully automated pre-concentration and sample cleanup is described as well as the detection of intact proteins using the Q Exactive HF mass spectrometer.

MATERIALS AND METHODS

The Thermo Scientific™ Vanquish™ Flex UHPLC system chosen for this setup, consisting of a quaternary low-pressure gradient pump (LPG) used as the loading pump and a binary high-pressure gradient pump (HPG) used as the analytical pump. The lower switching valve of the Vanquish Thermostated Column Compartment (VTCC) was used to switch between the sample loading and sample elution configuration (Figure 4). The loading pump was utilized to load the sample for two minutes on the trap column (Thermo Scientific™ MSPac™ DS-10 Desalting Cartridge) running an isocratic flow of 0.1% trifluoroacetic acid (TFA) in water at 300 µL/min. After the switching valve was switched to the elute position at 2 min, the trapping column was part of the analytical flow path and the HPG pump was delivering a 35 min 0.1% FA in water/acetonitrile gradient to elute the proteins from the trap column in back-flush mode and separate them on the Thermo Scientific™ MAbPac™ RP , 1 x 100 mm column. The column outlet was coupled to the Vanquish Variable Wavelength Detector (VVWD) and to the Q Exactive HF mass spectrometer equipped with a HESI-II probe in series. At 25.1 minutes, the switching valve was again switched to position 6_1 to allow the trap column to be equilibrated with the isocratic flow of the loading pump and to be ready for the next injection. If a setup with forward-flush is required to use the trap column also as a guard column, the position of the two capillaries of Port 4 and Port 5 on the switching valve (Figure 4) have to be swapped. The data were acquired with the Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2 SR4, and Thermo Scientific™ BioPharma Finder™ software, version 2.0, was used for data analysis.

RESULTS AND DISCUSSION

Using the described online SPE setup for protein samples allows a very fast and simple way to efficiently clean up, enrich, and separate proteins for LC-MS analysis. Figure 5 shows a representative total ion current (TIC) chromatogram for the baseline separation of a five protein mixture. The sample was loaded to the trap column and the actual separation started at 2.0 minutes. The peak at 3.2 minutes shows the eluting TFA, retained to the trap column during the loading step, and other very polar compounds eluting from the analytical column.

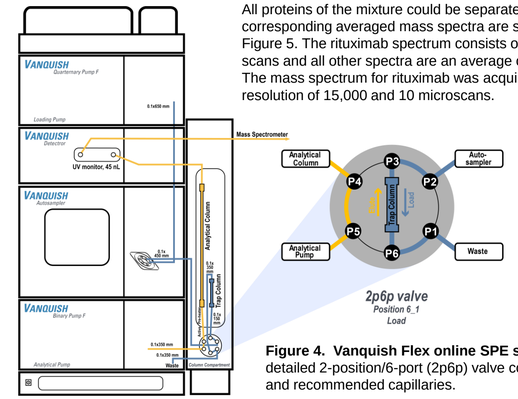


Figure 4. Vanquish Flex online SPE setup with the detailed 2-position/6-port (2p6p) valve configuration and recommended capillaries.

All proteins of the mixture could be separated and the corresponding averaged mass spectra are shown in Figure 5. The rituximab spectrum consists of 15 averaged scans and all other spectra are an average of 87 scans. The mass spectrum for rituximab was acquired with a resolution of 15,000 and 10 microscans.

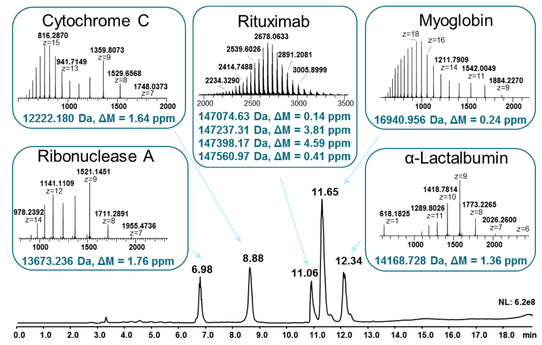


Figure 5. Analysis of a five protein mixture showing the separation and representative mass spectra for ribonuclease A, cytochrome C, rituximab, myoglobin, and α-lactalbumin A (total protein concentration, 600 ng) trapped on a MSPac DS-10 cartridge and separated on a MAbPac RP column.

For all other protein spectra, a resolution setting of 120,000 and 1 microscan were used to acquire isotopically resolved spectra for the determination of the monoisotopic mass. The intact mass of the five proteins in the mixture and the four most abundant glycoforms of rituximab are obtained after the deconvolution of the full MS mass spectra with the BioPharma Finder software. Theoretical and measured masses are shown in in Figure 5 with the individual mass deviation for each protein/isoform. The calculated mass deviation for all proteins is below 5 ppm and demonstrates the remarkable mass accuracy of the Q Exactive mass spectrometer.

TRADEMARKS/LICENSING

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