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Middle-up quantification of therapeutic monoclonal antibodies in human plasma with two dimensional liquid chromatography high resolution mass spectrometry: Adalimumab as a proof of principle

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ABSTRACT

Next generation human therapeutic monoclonal antibodies (t-mAbs) are harder to quantify with the widely used bottom-up tryptic digestion method. Due to their homology with endogenous immunoglobulins, there is a lack of unique and stable 'signature' peptides that can be targeted. Middle-up two dimensional liquid chromatography high resolution mass spectrometry (2D-LC-HRMS), targeting the entire light chain, was examined as an alternative. Adalimumab (ADM) was successfully quantified in human plasma after Melon® Gel sample purification, followed by orthogonal separation on a weak cation exchange (WCX) and reversed phase column. Charge and hydrophobicity were used to separate ADM from the polyclonal immunoglobulin background. HRMS with its high resolution and exact mass was able to isotopically resolve the ADM light chain and to provide another separation dimension on the basis of mass to charge ratio. Using the targeted single ion monitoring (T-SIM) with multiplex (MSX) option, three ADM light chain precursors, 2341.80, 2129.00, and 1951.68 m/z, and one internal standard precursor 2146.39 m/z, were measured simultaneously. The Melon® Gel sample purification was found to be very efficient in removing plasma proteins that would otherwise interfere with chromatographic separation and ionization. The linearity of the method for the analysis of ADM was excellent (R^2=0.999) between 1 – 128 mg/L with an LLOQ signal to noise ratio (S/N) of 10. Within-run and between-run precision and accuracy were in concordance with the EMA guideline. Cross-validation of the 2D-LC-HRM method with the standard peptide LC-MS/MS method showed a good agreement (R^2 = 0.86) between the methods. However, there was a bias present, possibly due to charge variant ADM formation over time. Since the presented 2D-LC-HRMS method is able to measure only the native form of ADM, it is able to provide a measure of the active form of ADM in patients.

Introduction

The quantification of therapeutic monoclonal antibodies (t-mAbs) by liquid chromatography- tandem mass spectrometry (LC-MS/MS) has grown tremendously in recent years [1–5]. In quantitative proteomics, the principle used predominantly, is based on a bottom-up approach targeting a 'signature' peptide of the t-mAb between 6 and 20 amino acids long. Generally, after sample purification, trypsin digestion is performed and tryptic peptides are separated with liquid chromatography (LC), and a signature peptide is analyzed by tandem mass spectrometry (MS/MS). This principle has proven to be very successful, providing enhanced selectivity, multiplexing ability, and a wider linear dynamic range compared to the traditional enzyme-linked immunosorbent assays (ELISA). Furthermore, relatively inexpensive reagents can be used for the analysis and methods allow the use of an internal standard (IS) which improves method precision significantly compared to ELISA.

For fully human or humanized t-mAbs this approach is sometimes not feasible due to a lack of unique signature peptides in the molecular structure [6]. This can be circumvented by choosing a selective sample preparation, on the basis of immunoaffinity purification, followed by mass spectrometry [7–10]. These targeted sample purifications methods use anti-idiotypic antibodies or ligands to bind the t-mAb selectively, thereby allowing isobaric in-

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terfering endogenous immunoglobulins (IgG) to be washed away. However, anti-idiotypic antibodies or ligands are expensive, the sample preparation time is long, and the method development is lengthy due to the numerous steps that need to be optimized. Furthermore, for newly developed t-mAbs, these anti-idiotypic antibodies or ligands are sometimes not commercially available. Therefore, there is a need for alternative mass spectrometry methods to quantify t-mAbs.

Recently, a middle-up quantification approach, which targets a larger portion of the protein, has shown great potential [11–13]. With this approach, the t-mAb is quantified by high resolution mass spectrometry (HRMS) targeting the light chain of the molecule, which is comprised of approximately 210 amino acids. This approach has a significantly higher chance of providing a unique amino acid sequence, and corresponding m/z value, that can be targeted with HRMS. However, since these human t-mAbs contain light chains with identical framework regions to patients own IgG, they are very similar to each other in terms of hydrophobicity leading to similar retention profiles on a reversed phase (RP) column. The resulting co-elution of light chains leads to ionization suppression and isobaric interferences, which negatively effects method sensitivity of the HRMS method. Therefore, we developed and validated an orthogonal two-dimensional liquid chromatography in combination with high resolution mass spectrometry (2D-LC-HRMS) method for the separation and quantification t-mAb in human plasma, using the fully human monoclonal antibody adalimumab (ADM) as proof of principle.

1. Materials and methods

1.1. Chemicals and reagents

ADM (Adalimumab; Humira) was obtained from AbbVie (Hoofddorp, The Netherlands) as a solution of 40 mg/0.8 mL. IFX (Infliximab; Remicade®) 10 mg/mL and GLM (Golimumab; Simponi®) 100 mg/ml were obtained from Johnson and Johnson (NJ, United States), RTX (Retuximab; MabThera®) 10 mg/mL and EMZ (Emicizumab; Hemlibra®) 150 mg/mL from Roche (Bazel, Zwitserland), CTX (Cetuximab; Erbitux®) 5 mg/mL from Merck (Darmstadt, Germany), DPL (Dupilumab; Dupixent®) 150 mg/mL from Sanofi Genzyme (MA, United States), DNX (Dinutuximab; Isquette®) 4.5 mg/mL from Rentschler Biotechnologie GmbH (Laupheim, Germany), VDZ (Vedolizumab; Entyvio®) 60 mg/mL from Takeda (Tokio, Japan). Kiovig® was obtained by Baxalta (Lessines, Belgium). Stable isotopically labeled (SIL), lysine [13C6,15N2] and arginine [13C6,15N4], IS ADM with mono isotopic mass of 23,591.609 g/mol for completely reduced light chain, was obtained from Promise Advanced Proteomics (Grenoble, France) as 10 µg/10 µL solution and was stored in -80 °C. Melon® Gel purification kit with product number 45,214 was obtained from Thermofisher Scientific (Waltham, MA, USA). Protein A magnetic beads were obtained from Promega (Madison, WI, USA). All other reagents, salts, and LC-MS grade mobile phase solvents were obtained from Sigma-Aldrich.

1.2. Preparation of standards, internal standard and QCs

The highest standard ADM 128 mg/L was prepared from ADM (50 mg/mL) stock solutions by diluting in drug free pooled human plasma. Aliquots were stored in Eppendorf tubes at -80 °C. Standards at concentrations of 1, 2, 4, 8, 16, 32, 64, and 128 mg/L were prepared from the stock solution by serial dilution in drug free pooled human plasma. IS SIL ADM working solution (10 mg/L) was prepared by dilution in PBS (0.1% Tween) and stored at -80 °C. Quality Control samples (QCs) at the lower limit of quantification (LLOQ) (1 mg/L), QC low (5 mg/L), QC med (50 mg/L), and QC high (100 mg/L), were prepared using a separate ADM stock solution and pooled plasma of a different batch. Aliquots were stored at -80 °C.

1.3. Instrumentation and chromatographic conditions

Sample purification was performed on an Eppendorf ThermoMixer C (Hamburg, Germany).

All measurements were performed on a vanquish LC coupled to a QExactive high resolution mass spectrometer from Thermofisher Scientific (Waltham, MA, USA). The vanquish system was equipped with 2 binary pump H modules, a diode array detector FG, and a 2-p 6-p 150 MPa bio 6036.156 valve to perform heart-cut injections. For the first separation dimension a ProSwift® WCX-1S 4.6 × 50 mm column was used, and for the second separation dimension a MAbPac® RP 2.1 × 10 mm was used, both from Thermofisher Scientific (Waltham, MA, USA). The WCX column was maintained at room temperature (25 °C) and the RP column was maintained in the column oven at 80 °C. The mobile phases for WCX were: (a) 10 mM phosphate buffer pH 7.6; (b) 10 mM phosphate buffer pH 7.6 and 1 M NaCl. The UHPLC gradients in minutes per percentage of mobile phase B were 0.0 (min)/0 (% B), 1.0/0, 5/50, 7/100, 8.00/0, and 10.00/0 with a flow rate of 1 mL/min. The mobile phases for the RP were: (a) water with 0.1% formic acid (FA); (b) 10% isopropyl alcohol, 1% FA in acetonitrile (ACN). The UHPLC gradients in minutes per percentage of mobile phase B were 0.0 (min)/20 (% B), 2.0/20, 6/26, 6.5/26, 9.9/60 and 10.00/20 with a flow rate of 0.5 mL/min.

The MS was operated in positive mode with a spray voltage of 3.5 kV, Capillary temperature 300 °C, Aux gas heater temp 300 °C, Aux gas flow rate 15 Arb, sheath gas flow rate 50 Arb, sweep gas flow rate 0 Arb, and S-Lens RF level 65. T-SIM mode with multiplexing was used with in-source CID 40 eV, resolution 140,000, AGC target 2E5, maximum IT 125 ms, isolation window 2.0 m/z, and scan range 1900 to 2400 m/z. The inclusion list contained three precursor charge states +10, +11, and +12 for ADM with the following masses 2341.80, 2129.00, and 1951.68 m/z respectively, and +11 precursor for SIL ADM IS with 2146.39 m/z. A heart-cut injection was performed between 2.15 to 2.35 minutes runtime (Fig. 1).

1.4. Sample preparation for LC-MS/MS analysis

The Melon® Gel sample preparation was performed by adding 120 µL purification buffer to a prewashed Melon® Gel slurry of 100 µL pipetted in a 96-well filter plate. Then, 10 µL standards or samples were added to the Melon® Gel slurry, followed by 10 µL IS SIL ADM 10 mg/L. After mixing for 15 min at 1000 rpm, the samples were centrifuged for 1 min at 1000 g to collect the purified samples. Then 100 µL TRIS 100 mM pH 7.5 containing 25% glycerol was added to stabilize the eluted immunoglobulins and 5 µl TCEP 100 mM was added to reduce the disulfide bonds. The samples were gently mixed at 1200 RPM for 30 min at room temperature. Finally, 20 µL sample was injected on the 2D-LC-HRMS.

1.5. Mobile phase buffer

MES buffer 20 mM pH 6.5 and phosphate buffer 10 mM pH 7.6 were compared to determine the ideal mobile phase buffer for the t-mAb light chain separation. The performance of both buffers was examined with seven t-mAb's namely adalimumab (ADM), golimumab (GLM), dinutuximab (DNX), vedolizumab (VDZ), emicizumab (EMZ), dupilumab (DPL), and infliximab (IFX). These t-mAb's were diluted to a concentration of 0.1 mg/mL in TRIS buffer (100 mM pH 7.5) containing 25% glycerol, and reduced with 5 µL TCEP at 25 °C for 30 mins while gently mixing at 1200 RPM.

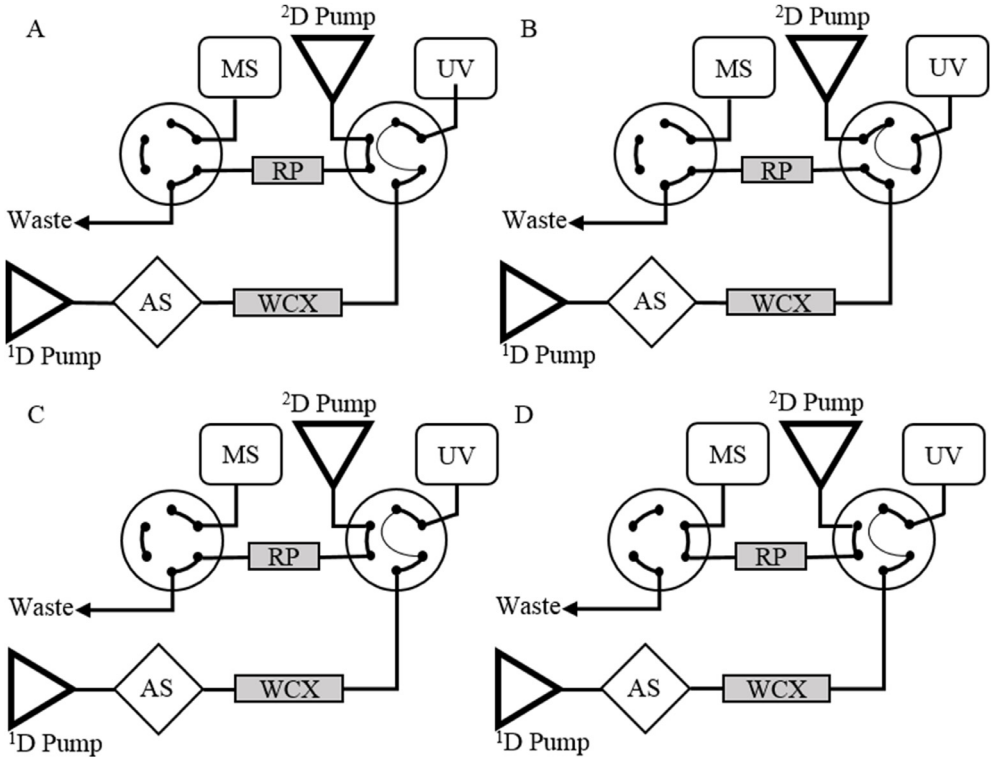

Fig. 1. Schematic picture of the 2D-LC-HRMS setup with (A) showing the settings for the first dimensional separation of ADM on the WCX column where the retention window for heart-cut loading of ADM in the second dimension is determined with an UV diode array detector (λ = 280 nm), (B) showing the settings for the heart-cut loading of the ADM fraction directly onto the RP column, (C) showing the removal of salts from the RP column, and (D) showing the second dimensional separation of the ADM light chain on the RP column with HRMS detection and quantification.

The purified IgG product Kiovig® was used for chromatographic comparison. The mobile phase gradient described in section 'instrumentation and chromatographic conditions' was used for both buffers.

1.6. Sample purification method comparison

Three different sample purification strategies were investigated for their efficiency in removing interfering proteins from the plasma. Melon® Gel, ammonium sulfate (AS) precipitation, and protein A purification were compared to each other and to an unpurified sample used as a reference.

In short, the Melon® Gel sample preparation procedure was performed as described above in Section 1.4 on an ADM standard of 1 mg/mL in duplicates.

AS precipitation was performed with the same ADM standard (1 mg/mL) by first adding 170 µL TRIS (50 mM, pH 8) containing 0.5% octyl glucoside (OG), then adding 10 µL standard in duplicates in a Lobind® Eppendorf tube. Immunoglobulin (IgG) fraction was precipitated by adding 200 µL saturated AS solution and mixed at 1600 rpm for 1 min and centrifuged at 4000 G for 5 min. The supernatant layer was decanted, and the pellet was dissolved in 240 µL TRIS (100 mM pH 7.5) containing 25% glycerol. Finally, 5 µL TCEP 100 mM was added to reduce the disulfide bonds and mixed at 25 °C for 30 min at 1200 RPM.

Protein A sample preparation was performed by adding 50 µL magnetic beads in two Lobind® Eppendorf tubes. The magnetic beads solution was removed by placing the Eppendorf tubes on a magnetic rack. The beads were washed three times with 200 µL PBS 0.1% Tween. The beads were suspended in 100 µL PBS 0.1% Tween solution. Then, 10 µL ADM standard (1 mg/mL) was added in duplicate and the solution was mixed for 1 hour to allow the IgG to bind to the protein A coated magnetic beads. The standard was washed three times with PBS 0.1% Tween and eluted with 100 µL 0.1% FA. After neutralizing with 10 µL 1 M TRIS, 140 µL TRIS (100 mM pH 7.5) containing 25% glycerol, was added followed by 5 µL TCEP 100 mM. The sample was reduced at 25 °C for 30 min at 1200 RPM.

The reference unpurified sample was obtained by adding 120 µL purification buffer in two Lobind® Eppendorf tubes. 10 µL ADM standard (1 mg/mL) was added followed by 100 µL TRIS (100 mM pH 7.5) containing 25% glycerol. Finally, 5 µL TCEP 100 mM was added and the sample was reduced at 25 °C for 30 min at 1200 RPM.

1.7. Method validation

Parameters such as within-run and between-run precision and accuracy, linearity, LLOQ, selectivity, matrix effect, carry-over, and stability were determined. These method performance criteria were evaluated following the EMA guideline for bioanalytical method validation [14]. In short, method precision and accuracy were determined by measuring QC LLOQ, QC low, QC med, and QC high in fivefold during three days. Selectivity was determined by measuring 10 blank human plasma samples. Matrix effect was tested by spiking 6 human plasma samples at two ADM levels of 5 mg/L and 100 mg/L. Carry-over was determined by injecting a blank after the highest standard. Stability testing was performed with QC low and QC high (n=5). Freeze and thaw stability testing was successfully validated for ADM in previous work [8] and was not included in the validation.

Stability at room temperature (20 °C) and 4 °C was tested during 3 days. Auto-sampler stability was tested by reinjecting validation samples containing the calibration curve and QC's after 7 days in the auto sampler at 5 °C.

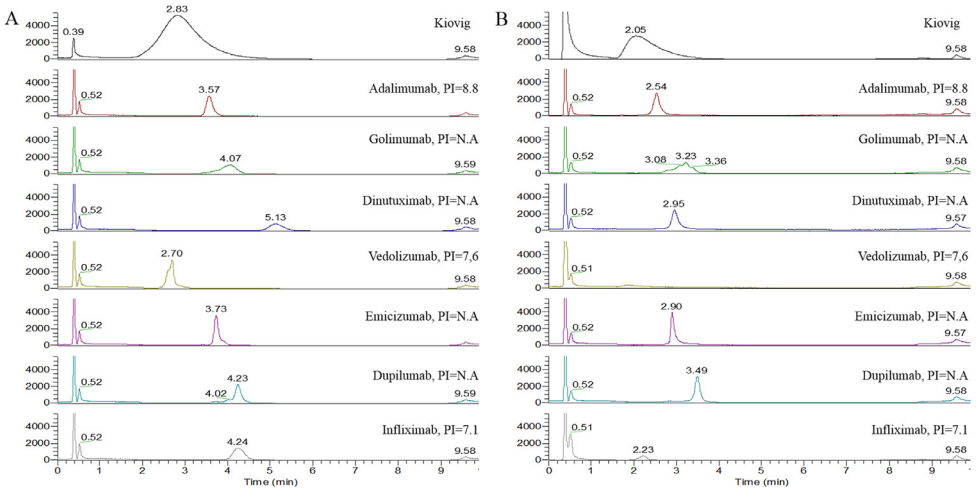

Fig. 2. Separation of seven t-mAbs (ADM, Adalimumab; GLM, golimumab; DNX, dinutuximab; VLZ, vedolizumab; EMZ, emicizumab; DPL, dupilumab, and IFX, infliximab) with MES buffer 20 mM pH 6.5 (A) and with phosphate buffer 10 mM pH 7.6 (B) on a Proswift WCX column with UV detection at 280 nm.

1.8. Cross-validation of the method

ADM EDTA plasma samples (n=37) used for routine therapeutic drug monitoring were stored at -80 °C prior to the 2D-LC-HRMS analysis. The ADM concentration ranged from 0 to 23 mg/L and the samples were previously quantified using an in house developed method based on trypsin digestion followed by LC-MS/MS analysis [8]. Results were compared using simple linear regression and Bland-Altman with software GraphPad.

1.9. Origin of charge variance in ADM treated patient

Experiments were performed on a patient's plasma sample with the highest difference (50%) in concentration between the middle-up and bottom-up method. This sample was used to determine the site of modification. The sample was highly purified using TNF-α pull down [8], reduced, and run on the RP column only. ADM standards were also analysed to determine the concentration. These samples were run on both the full scan mode, scanning range 1800–2500 m/z with resolution 140,000, as well as the T-SIM mode covering the three most abundant light chain precursors 10+, 11+, and 12+ as mentioned above.

2. Results

2.1. Mobile phase buffer

Retention on a WCX column is predominantly driven by the net charge of the protein and the salt gradient used. The protein net charge is in turn strongly dependent on its amino acid composition, which determines the PI, and on the mobile phase pH. MES buffer at pH 6.5 was able to retain all tested t-mAbs on the Proswift WCX column, including the entire polyclonal IgG fraction of Kiovig® (Fig. 2A). However, for the separation of ADM with an PI of 8.8 from other interfering polyclonal IgGs, phosphate buffer 10 mM pH 7.6 was found to be superior. Phosphate buffer at pH 7.6 was able to eliminate negatively charged and neutral polyclonal IgGs, which showed no retention on the column and eluted at the dead volume of 0.36 minutes (Fig. 2B). Here, Kiovig® represents the interfering polyclonal human IgG fraction that can be expected in patient's plasma. As can be seen from Fig. 2B, the IgG peak of Kiovig® at 2.05 minutes is reduced significantly since there are relatively more negative and neutral charged IgG's at pH 7.6 compared to MES buffer at pH 6.5. However, VDZ and IFX with a PI of 7.6 and 7.1, respectively, also elute at the dead volume and required a different mobile phase pH for optimal separation (Fig. 2B).

Since disulphide bond reduction was performed at room temperature (20 °C), the native conformation of the t-mAbs was retained and the light and heavy chain remained bound by internal non-covalent interactions. Therefore only one peak for each t-mAb was visible in the UV spectrum after WCX separation. However, when introducing the t-mAbs to the second dimension RP column, the high oven temperature of 80 °C in combination with high organic mobile phase, resulted in protein denaturation, and the light and heavy chain become detached and were separated based on their hydrophobicity.

2.2. Sample purification method comparison

Three sample purification strategies were examined to determine the best method to remove interfering plasma proteins. As can be seen in Fig. 3A, the chromatogram of the ADM standard 1 mg/mL spiked in human plasma, where no sample purification was performed, showed a high albumin and transferrin peak at the dead volume, which corresponds with a retention time of 0.38 minutes. ADM, with an isoelectric point (PI) of 8.8, was positively charged at the mobile phase pH of 7.6 and had a retention time of 2.29 minutes, whereas albumin and transferrin with a PI of 4.7 and 6.8 respectively, had a negative net charge and showed no retention on the WCX column. The corresponding zoomed in chromatogram in Fig. 3B has a high baseline between the retention window of 2.65 – 5.54 minutes compared to the Melon® Gel and Protein A sample purification. Within this retention window, other positively charged plasma proteins co-elute and in some samples showed interference with the second dimensional RP separation, leading to overlapping peaks (data not shown).

At a concentration of 50% saturated AS, immunoglobulins with a molecular weight (MW) of 150 KDa were precipitated, leaving behind small proteins, such as albumin and transferrin with a MW of 66 and 79 kDa, respectively, in the supernatant layer which were decanted and removed after centrifugation. The peak at 0.4 minutes in the AS purification chromatogram, is smaller compared to the no sample purification chromatogram (Fig. 3A). Here, albumin and transferrin, with molecular masses of 66 and 79 KDa, respectively, were predominantly eliminated. However, the ADM peak was visibly smaller, probably caused by the high AS concentration which was injected with the sample resulting in poor column binding which in turn led to a portion of AMD to elute faster.

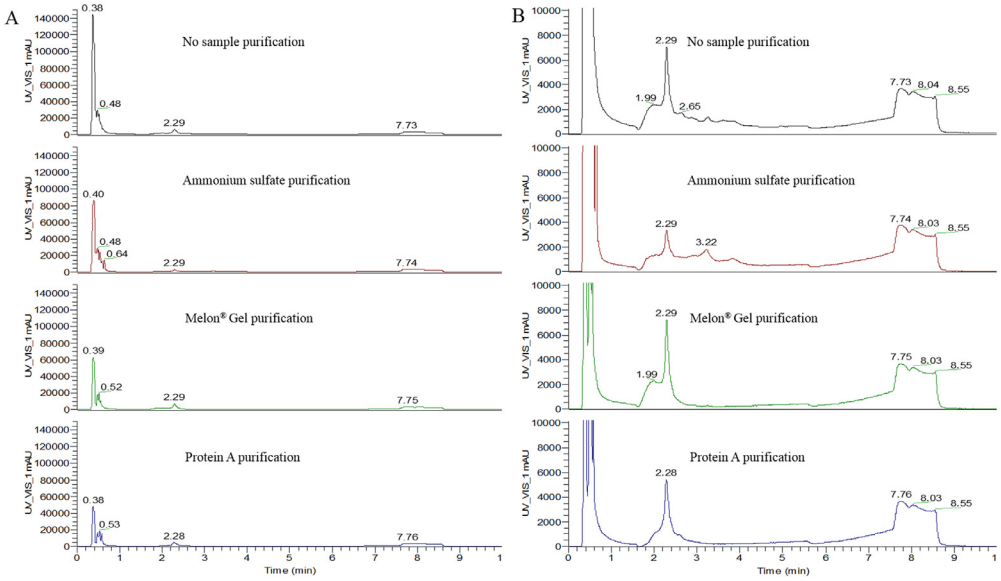

Fig. 3. UV chromatogram of adalimumab (ADM) standard 1 mg/mL spiked in human plasma and purified according the above mentioned conditions (A). Separation was performed on the ProSwift® WCX-1S column. (B) represents a zoomed in chromatogram of A. Top chromatograph (no sample purification) showing large albumin and transferrin peak at 0.38 min and ADM peak at 2.29 min. Ammonium sulfate purified sample chromatogram showing a lower albumin and transferrin peak at 0.38, a lower ADM peak at 2.29 min and remnant positively charged proteins between 2.65 – 5.54 min. Melon® Gel purified sample chromatogram showing even smaller albumin and transferrin fraction at 0.38 min, ADM signal intensity at 2.29 min is comparable to no sample purification chromatogram and at 1.99 min other immunoglobulin subclasses IgA, IgD, IgE, IgG_3, and IgM are visible. At the bottom, protein A purified sample chromatogram with the lowest albumin and transferrin peak at 0.38 min, ADM peak at 2.29, and reduced interference before and after ADM peak.

Furthermore, AS failed to remove plasma proteins between the retention window of 2.65–5.54 minutes.

Melon® Gel sample preparation binds albumin and transferrin, which are highly abundant in the plasma sample, and allows immunoglobulins to freely pass through the filter membrane. The sample preparation time is short, requiring only 15 minutes binding time, and the use of a 96-wells plate provides a high throughput. The dead volume peak at 0.39 minutes in the Melon® Gel chromatogram was almost as small as protein A purification. A high recovery was obtained with Melon® Gel, this was apparent by the similar peak heights found for ADM using Melon® Gel compared to the non-purified sample. Furthermore, plasma proteins between the retention window of 2.67–5.54 minutes were similarly eliminated. Protein A binds IgG's selectively through their FC regions and eliminated the most plasma proteins. The major difference between Melon® Gel and protein A sample purification chromatogram was the peak at 1.99 minutes. This peak presumably represents other immunoglobulin subclasses such as IgA, IgD, IgE, IgG_3, and IgM that were eluted together with IgG after the Melon® Gel purification, whereas with protein A purification they were largely eliminated. However, these overlapping proteins in the WCX chromatogram did not show any significant interference in the second dimension RP chromatogram.

Sample purification is essential since column performance can deteriorate faster when injecting unprepared samples. Furthermore, injecting high protein content onto the column can cause column overloading, leading to diminished resolution and poor chromatographic peak shape. In terms of sample purification efficiency, cost, and sample preparation time, the Melon® Gel seemed to provide the ideal balance and was therefore chosen for sample preparation.

2.3. Method validation

The LLOQ of ADM was 1 mg/L, with an average signal to noise ratio of 10 (Fig. 4). Linearity between 1 -128 mg/L was excellent with a R^2=0.999. Within-run and between-run precision and accuracy was well within the acceptance criteria for relative standard deviation (RSD) and bias of <15% (Table 1). LLOQ and QC high concentrations did not show any variations between the runs. For both parameters, the RSD and mean bias was <15% (Table 2). Here, similar signal intensity and results were obtained (data not shown). Benchtop stability (20 °C) and refrigerator (4 °C) stability were within acceptance criteria for accuracy and precision. Selectivity of the 10 individual blank human plasma samples were all <20% LLOQ.

During RP separation, a portion of the light chain denatures and precipitates on the column. This portion tends to elute during suc-

Table 1
Accuracy and precision validation data for adalimumab for QC's at LLOQ (1 mg/L), Low (5 mg/L), Medium (50 mg/L) and High levels (100 mg/L). Within-run data were based on 5 replicates and between-run data on 3 different days.

	Precision (% CV)			Accuracy (% bias)
QC	Within- run	Between-run	Overall	Overall
LLOQ	10.4	0	10.4	-3.6
Low	6.4	2.3	6.8	-6.5
Med	3.6	2.8	4.6	4.1
High	4.9	0	4.9	3.7

Table 2
Matrix effect test for adalimumab, 6 human plasma samples spiked at QC Low (5 mg/L) and QC High level (100 mg/L).

Sample nr	Measured [mg/L]	Bias	Measured [mg/L]	Bias [%]
1	4.88	-2.3	96.0	-4.0
2	4.58	-8.5	101.9	1.9
3	4.60	-8.0	104.1	4.1
4	4.76	-4.7	104.4	4.4
5	4.64	-7.2	99.1	-0.9
6	4.09	-18.2	93.0	-7.0
Average	4.6		99.7	
Stdev	0.3		4.6	
RSD	5.9		4.6	

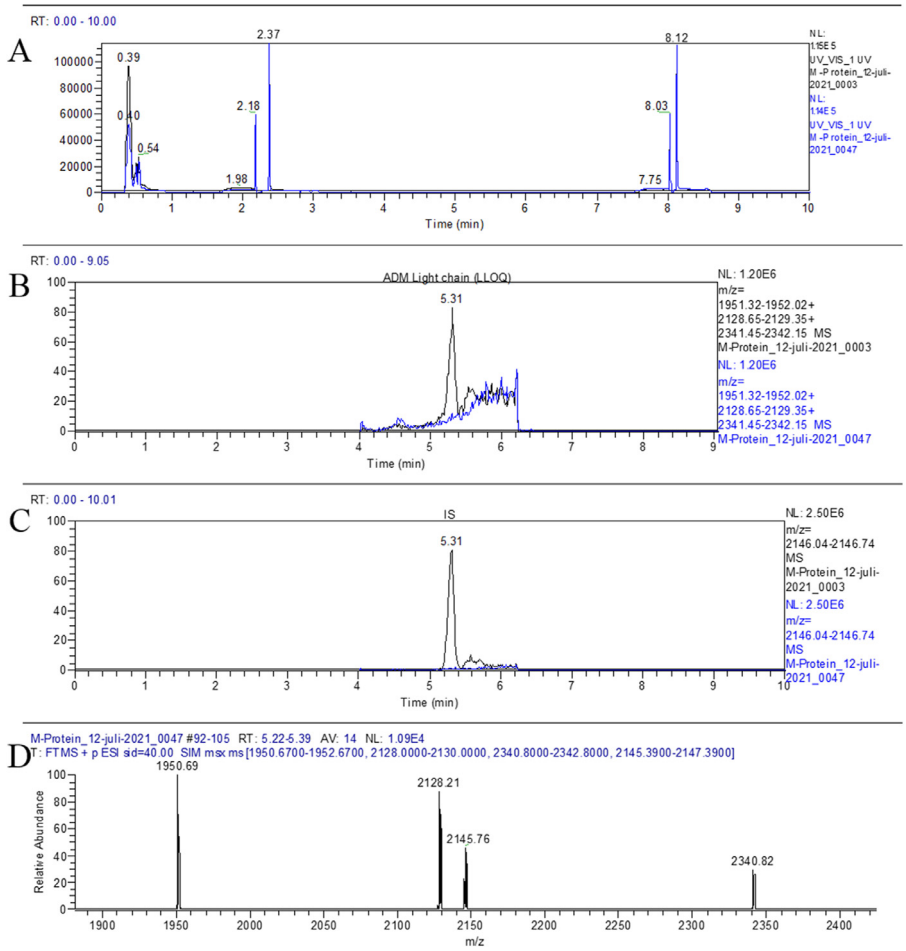

Fig. 4. LLOQ at 1 mg/L adalimumab (ADM) in black overlaid with blank human plasma in blue. Top view represents first dimensional chromatographic separation of standard 1 mg/L on a Proswift WCX column, heart-cut between 2.18 - 2.38 min measure with UV spectrometer (λ = 280 nm) (A). Followed by second dimensional chromatographic separation of ADM light chain on the MAbPac® reversed phase column (B) and the internal standard (IS) ADM light chain stable isotopically labeled (C). At the bottom the multiplexed spectral scan of ADM light chain and IS precursors measured on the Q Exactive HRMS (D).

cessive runs which lead to carry-over. The carry-over problem was reduced to an acceptable level (<0.2 mg/L, <20% LLOQ) by injecting three water blanks after a high ADM sample.

2.4. Cross-validation

Remnant EDTA plasma samples from 37 patients on ADM therapy were used for cross-validation of the 2D-LC-HRMS method with LC-MS/MS. As can be seen in Fig. 5, the middle-up 2D-LC-HRMS method results were on average 1.5 times lower compared to the bottom-up signature peptide LC-MS/MS method. Similar factors were also found in a previous comparison between ELISA and LC-MS/MS methods targeting signature peptides [8]. These differences were only seen in t-mAb treated patients and not in spiked plasma samples, which is in agreement with a previous publication where different ELISA assays were compared [15].

The reason for these differences could be due to the charge variants of ADM. In patients, a portion of ADM is modified, leading to the formation of an ADM fraction with a different PI and thus a different retention on the WCX column. Since a heart-cut injection was performed, this fraction would be missed and would thus result in a lower total (charge variant and native) ADM concentration. The same reason may also explain the lower ADM concentrations found with ELISA compared to LC-MS/MS. With ELISA, ADM is bound and measured by means of immunoaffinity interactions, which are primarily driven by charge interactions. Presumably, these charge variant ADM fractions could show poor interaction during binding and/or the detection stage in ELISA, which in turn would also result in lower total ADM concentrations in t-mAb treated patients.

2.5. Origin of charge variance in ADM treated patient

In contrast to the heavy chain, the light chain can be stable isotopically resolved with the Q Exactive® HRMS and is free from glycans and other isoforms. Therefore, a run was performed on a patient sample to determine whether the ADM light chain was modified through various pathways, such as oxidation, deamination, n-acetylation, and/or phosphorylation.

Results showed only one precursor of each charge state was visible at the retention time of 5.49 minutes, representing the native light chain form (Fig. 6). Furthermore, the sample was also quantified and the concentration of ADM, was similar to the LC-MS/MS signature peptide method. The good agreement of both methods is most likely due to the light chain was measured after RP separation only, since the sample was highly purified using a TNF-α pull down.

Therefore, for this sample, the modification is likely located in the heavy chain since 2D-LC-HRMS leads to two times lower results. Indeed, if the modification occurred in the light chain, then

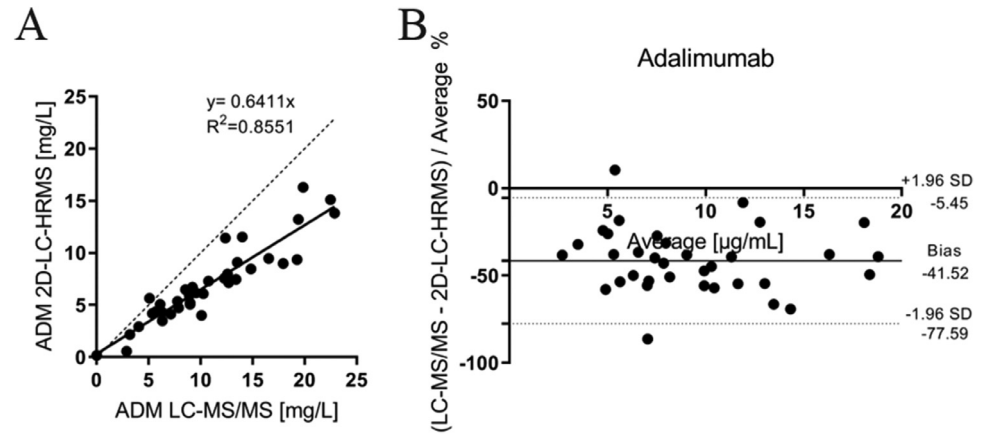

Fig. 5. Pearson correlation plot comparing LC-MS/MS bottom-up, signature peptide result with middle-up, 2D-LC-HRMS light chain result for patients treated with Adalimumab (A) (n=37). Bland-Altman plot of the sample results, two samples with results lower than the LLOQ were left out (B).

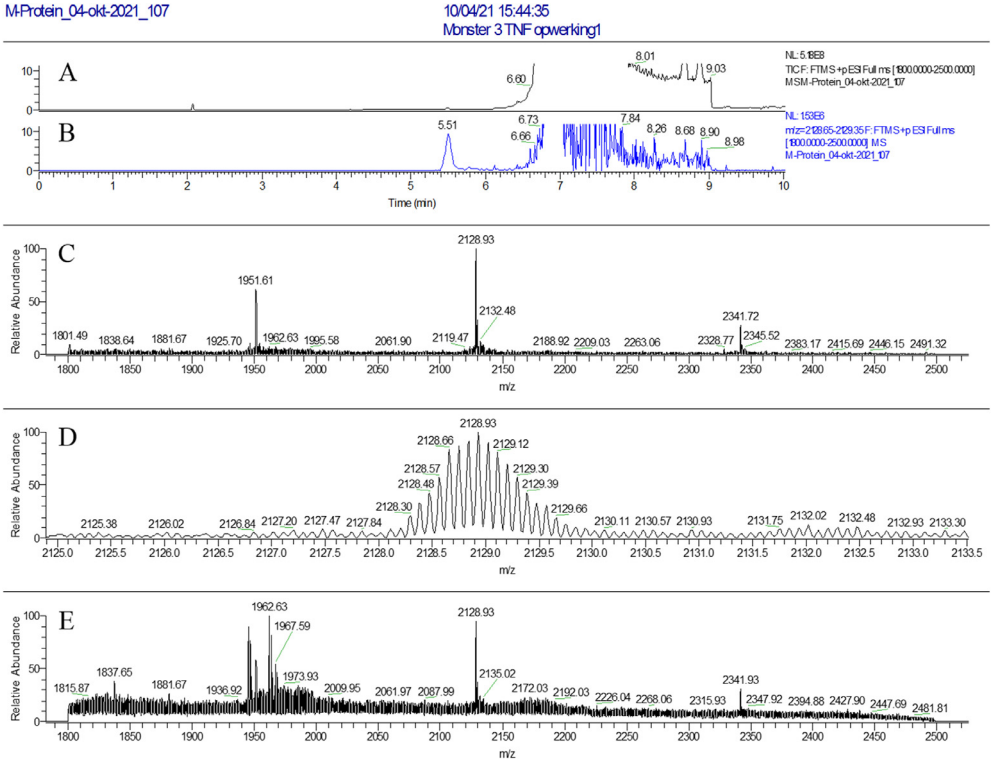

Fig. 6. TNF-α purified sample with 50% charge variant adalimumab (ADM). Chromatogram showing full scan 1800–2500 m/z view with no filters used (A). Chromatogram showing the full scan MS 1800–2500 m/z targeting the native form ADM light chain precursor 11+ at 2129 ± 0.35 m/z (B). Full mass spectrum 1800–2500 m/z of light chain peak RT 5.51 minutes (C). Zoomed in view of 11+ precursor (D). Full mass spectrum 1800–2500 m/z of all eluting peaks between the retention window of 2.135–6.11 minutes (E), no additional masses detected next to the light chain precursor 11+ (2129 m/z). Masses 1962.63 and 1945.61 are singly charged interference ions present through the run.

an equally high extra peak near the native form 2129 m/z would have been clearly visible in Fig. 6E. In vivo enzymatic processes similar to C-terminal lysine removal by carboxypeptidase B could be the cause of the differences observed [16]. However, it is important to note that the majority of C-terminal lysine is removed during the manufacturing processes [17]. Furthermore, the N-terminal residue of the heavy chain which consisting of glutamic acid would not result in change of charge upon in vivo cyclization to pyroglutamate [18]. Deamidation of the two -NG- groups found in the heavy chain could result in the formation of -DG- which would reduce the PI of ADM, resulting in faster elution on WCX.

3. Discussion and conclusion

A middle-up 2D-LC-HRMS method to quantify ADM was successfully developed and validated following the EMA guideline [14]. Different sample purification strategies, such as AS precipitation, Melon® Gel, and protein A sample purification were investigated. Melon® Gel was found to provide a good balance between performance, sample preparation time, and costs.

In contrast to MES buffer pH 6.5, phosphate buffer at pH 7.6 proved to be efficient in eliminating interfering negative charged and neutral immunoglobulins. MES buffer at pH 6.5 provided good

retention on the Proswift WCX column for most immunoglobulins, which could be useful for analyzing other t-mAbs with a low PI.

Cross-validation showed that ADM concentration in patients was a factor 1.5 lower when measured with 2D-LC-HRMS compared to LC-MS/MS, possibly due to the formation of a charge variant ADM in patients over time. 2D-LC-HRMS was able to separate and quantify native ADM concentrations, whereas the bottom-up signature peptide LC-MS/MS method can only quantify total ADM concentrations. The bottom-up LC-MS/MS method targets a much smaller portion of the entire t-mAb, which has a lower probability of being modified. This is comparable to ELISA assays, which also show lower concentrations of t-mAbs in treated patients compared to LC-MS/MS methods [8, 15]. Historically, the explanation for these differences was that there is a hypothetical to antigen or antibody bound ADM fraction that ELISA is not able to measure. Experiments performed in house, using acid dissociation on through plasma samples, did not show any significant amount of bound (either to antigen or antibody) ADM fractions. Confirming earlier findings by the Mayo Clinic [19], stating that "Although the comparator immunoassay theoretically measures the ATI-unbound fraction of infliximab, the equivalent quantitation suggests that the immunoassay may actually be measuring total infliximab". Hence, this does not explain the discrepancies found between 2D-LC-HRMS and the peptide LC-MS/MS method or for that matter LC-MS/MS and ELISA. On the other hand, in vivo modification of native ADM could lead to the formation of a charge variant ADM, which has a lower affinity to the binding/detecting antibodies used in ELISA, which would in turn lead to low recoveries and lower ADM concentrations. In vivo deamidation of trastuzumab has been described to lead to loss of recognition by ELISA antibodies [20]. Since it is unclear how active modified ADM is during the treatment, the presented 2D-LC-HRMS method, measuring only the native ADM form, provides a conservative measure of active ADM concentrations in patients on ADM treatment.

Notes

Compliance with ethical standards

Informed consent

Patient samples were drawn for routine care after obtaining informed consent.

Ethics committee

The use of anonymized remnant material drawn as part of the treatment protocol and with patient's informed consent was in accordance with University Medical Center Utrecht policy and ethical standards.

Declaration of Competing Interest

2D-LC system, columns, and Melon® Gel were provided by Thermo Fisher Scientific.

CRediT authorship contribution statement

Mohsin El Amrani: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft. **Kim C.M. van der Elst:** Supervision, Writing – review & editing. **Alwin D.R. Huitema:** Writing – review & editing. **Matthijs van Luin:** Supervision, Project administration, Resources, Writing – review & editing.

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