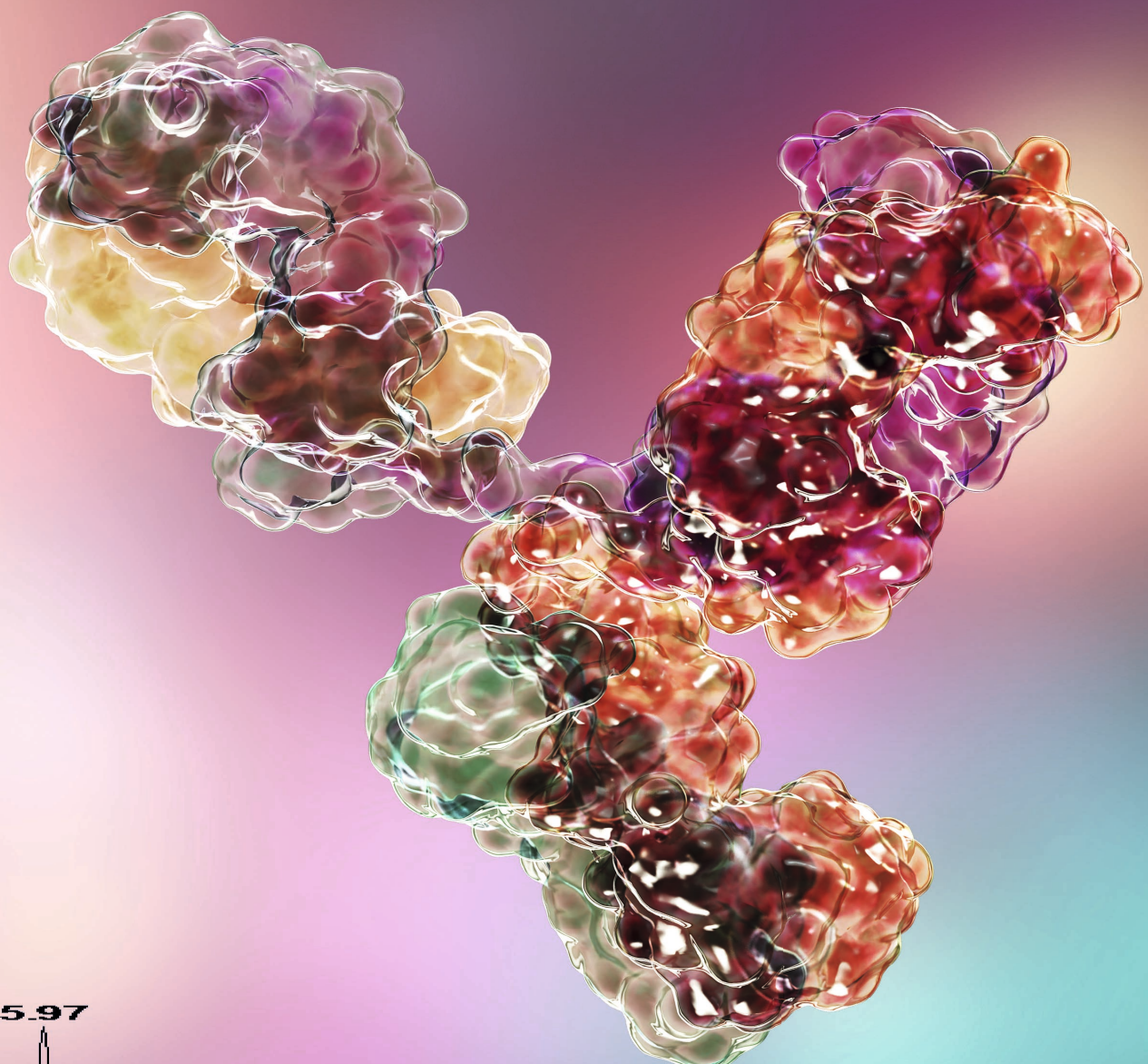
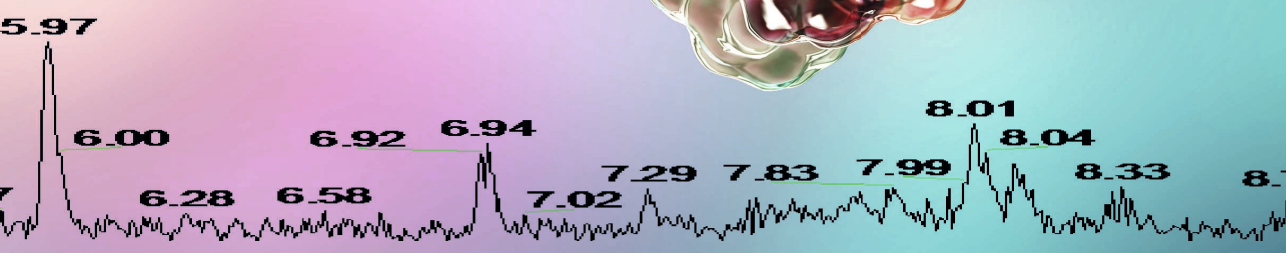
Quantification of therapeutic antibodies and endogenous proteins with LC-MS/MS

Mohsin El Amrani





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Het kwantificeren van therapeutische antilichamen en endogene eiwitten met LC-MS/MS

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. H.R.B.M Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 28 mei 2020 des middags te 2.30 uur

door

Mohsin El Amrani

geboren op 18 september 1978
te Utrecht

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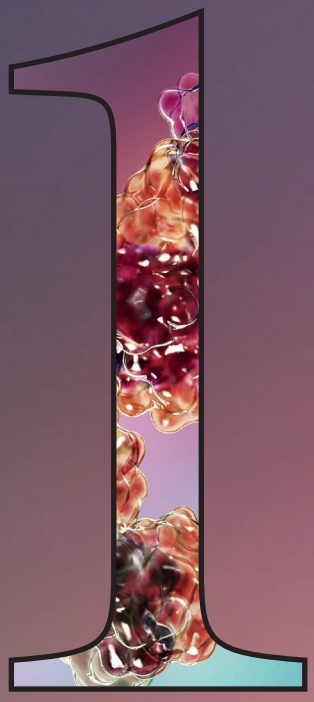

General introduction, scope and outline of the thesis

Background

Quantitative proteomics entails the quantification of endogenous and exogenous therapeutic proteins and plays an important role in disease diagnosis, pharmacokinetics (PK) research, therapeutic drug monitoring and biomarker discovery [1-3]. Therapeutic proteins are widely used in treatment of many diseases such as genetic disorders, inflammatory autoimmune diseases and cancer.

Genetic disorders are caused by gene mutations and lead to the expression of a wrongly sequenced protein that is unable to perform its designated task resulting in an overall underexpression of functional protein. Patients can be treatment for low protein expression through intravenous infusion with a recombinant form of the therapeutic protein [4-6]. Quantitative proteomics provides a means to diagnose the disease and its severity, study PK and to monitor the patient during treatment [7]. Moreover, quantitative proteomics is frequently used in biomarker discovery. Indeed, overexpression of proteins can be a good indicator of the presence of certain diseases. For example, elevated levels of prostate-specific antigen can be a sign for prostate cancer, elevated levels of C-reactive protein can be an indicator for heart disease and overexpression of tumor necrosis factor alpha (TNF-α) can be a sign of inflammatory autoimmune disease such as rheumatoid arthritis, Crohn's disease or ulcerative colitis [8-10]. Protein overexpression in some cases is treated by blocking the disease inducing protein, as in the treatment of patient with TNF-α overexpression with infliximab or adalimumab. During treatment, therapeutic drug monitoring of these therapeutic proteins is strongly recommended to determine drug plasma levels since low levels are associated with the development of anti-drug antibodies and poor treatment outcome [10]. These anti-drug antibodies can also be monitored in plasma and can be used to optimize patient treatment plan [11].

Furthermore, biomarker discovery on cancer cells can also lead to the identification of over-expressed protein which can be ideal targets for monoclonal antibody based therapy [12]. Example of these is the discovery of over-expressed human epidermal growth factor receptor 2 in certain types of breast cancer which can be treated with trastuzumab or over-expressed GD2 receptor on cancer cells in children with neuroblastoma which is treated with dinutuximab [13, 14].

Traditionally, the quantification of endogenous or exogenous therapeutic proteins is performed by ligand binding assays such as Enzyme Linked Immunosorbent Assay (ELISA) or Radioimmunoassay (RIA). However the selectivity of these assay relies solely on the ability of the capturing and/or detecting antibodies to correctly bind to the target protein. Furthermore, different ligand binding assays for the quantification of a specific protein would often generate different results depending on the specificity and avidity of the antibody

used in the test [15]. Therefore, due to the lack of standardization, comparing results from different centers is difficult to perform which could affect clinical decision making. Quantitative proteomics with liquid chromatography-tandem mass spectrometry (LC-MS/MS) can solve many of the above mentioned limitations found in ligand binding assay and provides additional advantages, such as enhanced sample through-put, linear dynamic range, precision, selectivity and multiplexing abilities [16-19].

Objectives of the thesis

The aim of this thesis is to provide insight to the development of LC-MS/MS methods for the quantification of endogenous proteins and exogenous therapeutic proteins such as monoclonal antibodies. These methods lay down the foundation of quantitative proteomics with LC-MS/MS and can serve as templates for future method development.

Outline of the thesis

Chapter 2: Therapeutic antibody quantification

Chapter 2.1 provides a tutorial and an overview of published methods for top down, middle down, and bottom-up quantitative proteomics and explains in six steps how to develop a LC-MS/MS method for the quantification of therapeutic proteins based on the popular bottom-up proteomics approach. Considerations for signature peptide selection are provided and critical method parameters are discussed for internal standard selection, chromatographic separation, sample purification, digestion and method validation. Chapter 2.2 provides an example of the selective purification of active infliximab in human plasma followed by LC-MS/MS quantification. In chapter 2.3, multiplexing is introduced for the simultaneous quantification of both adalimumab and infliximab providing higher sample through-put and ease of use through improved elution conditions and the elimination of the disulfide reduction step. In chapter 2.4, the T-cell binding polyclonal rabbit anti-thymocyte globulins are quantified on the basis of a selective purification with Jurkat T-cell line and a signature peptide originating from the constant chain. Finally, a generic purification step for monoclonal antibody determination on the basis of ammonium sulfate precipitation to eliminate the albumin fraction is introduced in chapter 2.5 for the quantification of dinutuximab in plasma. This method provides alternatives when anti-idiotypic antibodies or ligands to the target

protein are unavailable

Chapter 3: Endogenous protein quantification

Chapter 3.1 shows how competitive displacement in combination with enzymatic digestion and LC-MS/MS analysis can be used to quantify the neutralizing anti-infliximab antibodies in human plasma thus providing for the first time a way for LC-MS/MS to monitor development of neutralizing antibodies in patients. In the final chapter 3.2, coagulation FVIII in human plasma is quantified by first triggering the coagulation cascade, which frees FVIII from von Willebrand factor and selectively purifies active FVIII with camelid nanobodies followed by heat denaturation, trypsin digestion and LC-MS/MS analysis.

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Therapeutic antibody quantification

2.1

Six-step workflow for the quantification of therapeutic monoclonal antibodies in biological matrices with liquid chromatography mass spectrometry – A Tutorial

Mohsin El Amrani, Anouk A.M. Donners, C. Erik Hack, Alwin D.R. Huitema, Erik M. van Maarseveen

Analytica Chimica Acta, 1080 (2019) 22-34

Abstract

The promising pipeline of therapeutic monoclonal antibodies (mAbs) demands robust bioanalytical methods with swift development times for pharmacokinetic studies. Over the past decades ligand binding assays were the methods of choice for absolute quantification. However, the production of the required anti-idiotypic antibodies and ligands limits high-throughput method development for sensitive, accurate, and reproducible quantification of therapeutic mAbs.

In recent years, high-resolution liquid chromatography tandem mass-spectrometry (LC-MS) systems have enabled absolute quantification of therapeutic mAbs with short method development times. These systems have additional benefits, such as a large linear dynamic range, a high specificity and the option of multiplexing.

Here, we briefly discuss the current strategies for the quantification of therapeutic mAbs in biological matrices using LC-MS analysis based on top-down and middle-down quantitative proteomics. Then, we present the widely used bottom-up method in a six-step workflow, which can be used as guidance for quantitative LC-MS/MS method development of mAbs. Finally, strengths and weaknesses of the bottom-up method, which currently provides the most benefits, are discussed in detail.

Introduction

Therapeutic monoclonal antibodies (mAbs) nowadays are widely accepted as valuable treatment options for patients suffering from a variety of diseases, particularly in the areas of oncology and immune diseases. At present, 76 mAbs have been granted market authorization by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) and are now commercially available for therapeutic use [1]. Judging from drug pipelines this number is set to grow considerably in the near future [2]. Therapeutic mAbs target pathological processes with high specificity and concomitantly lead to fewer side effects compared to conventional small molecule based therapies [3]. Furthermore, due to the high molecular weight of mAbs, the clearance pathway is not by renal elimination after hepatic enzyme metabolism but rather by proteolytic catabolism, receptor-mediated uptake and degradation, and sometimes by the catabolic pathway of their molecular target. Two thirds of mAbs are salvaged from degradation by binding to the protective neonatal Fc-receptor (FcRn) particularly on endothelial cells, which extends their elimination half-life to ~18–21 days [4].

MAb production and design has made great strides from the early discovery in 1975 by Kohler and Milstein [5]. The progression from murine mAbs (1975) using hybridoma technology to chimeric mAbs (1984) using recombinant DNA techniques to humanized mAbs (1988) using complementary-determining region (CDR) grafting and finally to fully human mAbs (1994) using phage display or transgenic mice took less than 20 years [6-9]. These steps were essential to reduce the risk of anti-drug antibodies (ADA) development and allergic reactions associated with first generation mAbs [10-12]. In fact, additional requirements from the EMA, Food and Drug Administration (FDA) and World Health Organization (WHO) for the evaluation and monitoring of immunogenicity of new biopharmaceuticals were mandated as part of regulatory approval, together with a rigorous post-authorization pharmacovigilance with product-level traceability for of all biopharmaceuticals [13-17].

The discovery of new therapeutic targets and the high treatment efficacy of biopharmaceuticals accelerated the development of novel mAb-based therapies [16, 18]. For this purpose, bioanalytical methods were necessary to facilitate the required preclinical pharmacokinetic (PK) studies. In addition, therapeutic drug monitoring of mAbs concentrations can highlight accelerated drug clearance in patients which is indicative of ADA development and potential loss of drug response. Traditional bioanalytical methods such as ligand-binding assays rely on an anti-idiotypic antibody or a ligand with high avidity towards the therapeutic protein of interest. However, the development of such antibodies is notoriously difficult and time-consuming [19-21]. Therefore, advances in analytical techniques were essential to attain shorter method development times, which is why liquid chromatography-tandem mass spectrometry

(LC-MS/MS) has received increasing interest as an alternative method for quantification over the last decade. Following strength and weaknesses analysis of ligand binding assays, this tutorial systematically addresses bioanalytical methods to quantify therapeutic mAbs in biological matrices using LC-MS. Three main branches of quantitative proteomics using top-down (intact), middle-down (semi-intact) and bottom-up (signature peptide) strategies are briefly explored. Finally, the most widely used bottom-up quantification strategy via signature peptide is chronologically discussed in a general workflow where strengths and weaknesses of each step are extensively explained.

Ligand binding assay

Enzyme-linked immunosorbent assay (ELISA) is arguably the most widely used form of ligand binding assay (LBA), because of its high sensitivity, ease of use, and low instrumental costs [22, 23]. Moreover, due to the spectrophotometric detection principle, ELISA offers a safer alternative with a high ease of use compared to historically used radioimmunoassays which require special facilities and operators trained to handle radioactive material [24]. In general, ELISA methods are based on the quantification of the target protein (antigen) using strategies, such as direct, indirect or sandwich type ELISA (Fig. 1) [25-27]. In the final step, quantification is performed by adding a substrate solution, often tetramethylbenzidine (TMB), which is gradually oxidized by horseradish peroxidase (HRP) to a colored product.

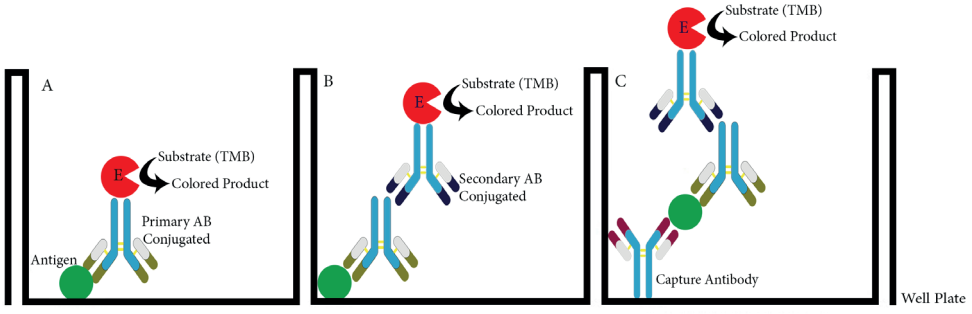


Figure 1: A) Direct ELISA, B) Indirect ELISA and C) Sandwich ELISA, (TMB= Tetramethylbenzidine)

To improve sensitivity the use of a polyclonal secondary antibody in indirect or in sandwich type ELISA can help boost the signal intensity [28]. Alternatively, polymerized HRP can be used to increase sensitivity. Background staining is a very common challenge in these assays, and is often caused by non-specific interactions of either the primary or the secondary

polyclonal antibody used [29][17, 32]. The choice of binding strategy can help minimize the risk of non-specific interactions. For example, in sandwich type ELISA, an additional anti-idiotypic antibody binding the antigen at a different epitope provides higher specificity [30]. The reliance of ELISA methods on these specific anti-idiotypic antibodies or ligands can lead to lengthy method development times [31] and higher consumables costs compared to LC-MS/MS methods. Furthermore, ELISA's allow for one component analysis in one specific bio-matrix, while LC-MS/MS methods allows for multiplexed measurement of multiple therapeutic and endogenous proteins in various bio-matrices [32-34] and due to the narrow linear dynamic range in ELISA, accurate quantification may require multiple sample dilutions thus limiting sample throughput.

Importantly, the validation acceptance criteria for ELISA are less stringent in comparison to LC-MS/MS methods [35, 36], mainly because ELISA methods cannot incorporate internal standards to correct for binding efficiencies influenced by sample matrix or component loss due to binding and washing steps. Finally, different results can be obtained with different ELISA assays as was demonstrated by Vande Casteele in the comparison of three commercially available ELISA kits for infliximab and anti-infliximab quantification, stating that comparison of drug levels and ADA monitoring is hampered by lack of standardization [37]. Inter-assay variability makes it difficult for clinicians to compare results from other centers and invalid measurements affects decisions made in patient's diagnosis and treatment.

Top-down, middle-down and bottom-up quantitative proteomics

Quantification with LC-MS offers various advantages over LBA and over the years various LC-MS strategies have been explored and reported. Three main strategies are discussed in detail namely top-down, middle-down and bottom-up proteomics. The flow chart in figure 2 depicts the decision making process for the preferred strategy for quantification based on factors, such as the intended assay specifications in terms of sensitivity and selectivity and the applied instrumentation and materials. For therapeutic drug monitoring of trough levels, in most cases a LLOQ of 1 mg/L would suffice [38]. This would allow for the quantification of the peptide using LC-MS/MS or intact after selective purification using various instruments such as LC-HRMS, LBA or LC-FLD. However, for pharmacokinetic applications more sensitive assays are regularly required (e.g. 100 µg/L or less) to characterize the terminal elimination phase. Therefore, in most cases only LC-MS/MS using bottom-up proteomics to quantify the signature peptide or LBA after selective purification with anti-idiotypic antibodies would be suitable (Fig. 2).

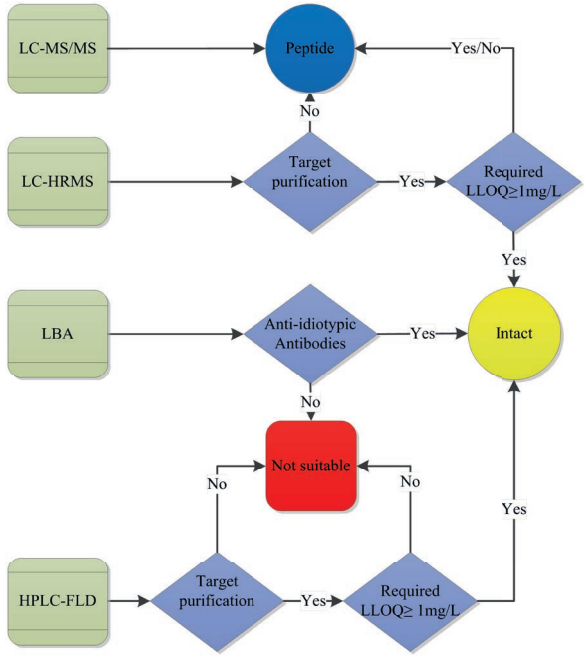


Figure 2: General recommendation for therapeutic mAb quantification depending on instruments and materials available and on assay requirements; Quantification on the basis of peptide (bottom-up) using LC-MS/MS or LC-HRMS and intact on the basis of middle-down and top-down strategies with LC-HRMS or intact on the basis of LBA, HPLC-FLD. (HRMS= High resolution MS; LBA= Ligand binding assay; FLD= Fluorescence detection)

Bottom-up quantitative proteomics is preceded by denaturation and enzymatic digestion of the therapeutic mAb which releases numerous peptides of different chain lengths. Peptides that are unique for the mAb (signature peptides) are selected for measurement. These peptides are easily and efficiently separated using a standard reverse-phase HPLC system and thereafter quantified on a standard triple quadrupole mass spectrometer. In general, the chromatographic peak shape of the peptides is more symmetrical compared to the peak shape of proteins due to fewer secondary interactions on the stationary phase.

Top-down and middle-down quantitative proteomics is based on the measurement of intact and semi intact proteins. Large proteins such as mAbs can shift to high charge states during electrospray ionization yielding a mass to charge (m/z) ratio within the working range ~1800 – 4000 of a high resolution mass spectrometers (HRMS) such as Orbitrap or Time-Of-Flight (TOF). These methods do not require protein unfolding and enzymatic digestion which can be challenging and time-consuming to optimize. However, top-down and middle-down

methods do have some limitations. Firstly, targeted sample purification with anti-idiotypic antibodies or ligands is necessary due to structural similarities between the therapeutic mAb and endogenous IgG. Secondly, the required HRMS apparatus is expensive compared to the triple quadrupole mass spectrometer. Finally, attaining the required lower limit of quantifications (LLOQ) can be challenging because of wider precursor charge distribution, broadened chromatographic peak shape of large proteins, and mAb glycoform heterogeneity [39-41]. Some additional steps can be implemented to gain higher signals and thus lower LLOQ's. For example, human immunoglobulin G1 in rat serum was successfully quantified with LC-HRMS after target specific purification with anti-idiotypic antibodies followed by deglycosylation [32]. Here, a remarkable LLOQ of 0.1 µg/mL was achieved through this strategy by utilizing a high sample volume (50 µL) in combination with high volume injection (60 µL) and 1 mm diameter analytical column.

Top-down quantification can also be performed by HPLC coupled to fluorescence detector (FLD). For example, intact trastuzumab, bevacizumab and infliximab in human serum were successfully quantified by HPLC-FLD after targeted purification [42, 43]. Unlike HRMS methods, FLD methods are not affected by signal dilution caused by charge distributions. However, fluorescents measurements have a low specificity. Most proteins have similar excitation and emission spectra which result in noisy and overlapping chromatographic peaks. Furthermore, because of the lower sensitivity, a higher sample volume (>100µL) is required which limits its applicability.

Middle-down strategies can also be used to reduce precursor charge distribution found in intact analysis. Here, only a portion of the mAb is measured such as the light chains after dithiothreitol (DTT) reduction, or Fab regions after limited Lys-C digestion [44, 45]. In contrast to intact mAb measurement, these regions are smaller and are usually free from glycan chains, leading to fewer precursor ions resulting in an increase in signal intensity of the mAb. As can be seen from figure 2, peptide level quantification via LC-MS/MS or LC-HRMS instruments have low requirement and are thus frequently employed. This approach has been extensively used for numerous mAbs with great success. However, for some fully human/humanized therapeutic mAbs, quantification via signature peptide can be challenging because of the human polyclonal serum background [46]. In these situations, a targeted purification followed by intact or peptide level quantification might be preferred.

Bottom-up quantification

Currently, bottom-up quantification of mAbs in biological matrices using signature peptides is

the most common approach. This principle offers fast, easy and flexible method development with high detection sensitivity using standard triple quadrupole mass spectrometers. Generally, these published methods share similarities as they all include steps depicted in figure 3.

Figure 3: General workflow to develop quantitative LC-MS/MS method to measure therapeutic mAbs

The major differences between these methods are the selection of internal standard, sample purification and digestion conditions (Table 1). The merits and drawbacks of various options in each steps of method development will be discussed in next sections.

Table 1: Published methods for peptide level quantification of therapeutic mAbs with LC-MS/MS

Sample type			Internal standard used				
Rodent	Monkey	Human	Analogue protein	SIL peptide	SIL protein	Extended SIL peptide	Dimethyl labeled
[33, 47-53]	[21, 54-64]	[34, 65-85]	[47, 63, 76, 79-81, 83]	[34, 49, 53, 54, 57, 60, 64, 67-71, 73, 75, 78, 85]	[21, 33, 48, 51, 52, 56, 59, 62, 72, 74, 77, 82]	[58, 61, 65, 73]	[55]
Sample purification							
Not performed (Whole digest)	Albumin depletion	Pellet Digestion	Protein A	Protein G	Anti-human FC antibody	Anti-Idiotypic antibody	Ligand
[21, 47, 55, 60, 73, 78]	[33, 54]	[49-52, 57, 58, 61, 63, 64, 66, 67, 75, 85]	[45, 53, 68-71, 80, 84]	[65, 76, 81]	[48, 56, 59, 60, 62]	[34, 65]	[72, 74, 77, 79, 82, 83]
Digestion (denaturation)				Digestion (reduction & alkylation)			
Guanidine	Urea	Surfactant	TFE	Not performed	TCEP and IAA	DTT and IAA	DTT
[47]	[21, 33, 48, 54-56, 59, 74, 82]	[34, 50, 60, 62, 73, 78, 85]	[66]	[49, 51, 57, 64, 67, 71, 75, 82, 83]	[33, 48, 60, 65, 77]	[21, 34, 47, 50, 52-56, 58, 59, 61-63, 66, 73, 76, 78, 80, 81, 85]	[72, 79]
Post digest SPE cleanup		Mass spectrometer			LLOQ method performance		
RP	Ion exchange	Triple Quad	Q-Trap	HRMS	≤0.5mg/L	≤1 mg/L	>1mg/L
[33, 47, 48, 75, 78]	[47, 52, 60, 61, 63, 73]	[21, 34, 47, 50, 51, 53, 55, 56, 58-62, 64-68, 71-76, 79-81, 85]	[33, 48, 49, 54, 57, 63, 78, 82]	[52, 77, 83]	[34, 47, 48, 50, 53, 56, 59, 62, 65, 67, 71, 77, 84, 86]	[49, 52, 55, 57, 65, 68, 74, 75, 81-83, 85]	[21, 33, 51, 54, 58, 60, 63, 64, 66, 73, 76, 78, 80]

SIL = stable isotopically labeled
TFE = Trifluoroethanol
TCEP = tris(2-carboxyethyl)phosphine
IAA = Iodoacetamide
DTT = Dithiothreitol
SPE = Solid phase extraction
RP = Reversed phase

Signature peptide selection

The first step in method development is selection of unique signature peptides. The peptide sequence of the therapeutic mAb is essential for this step. Sequences of approved therapeutic mAb can be found in the Immunogenetics Information system® (http://www.imgt.org/) or in Drugbank (http://www.drugbank.ca). For the quantification of chimeric mAbs in human serum, tryptic peptides from the entire variable region can be chosen and targeted. However, for human or humanized mAbs the choice is limited to the complementarity-determining regions (CDRs) of which there are six in the variable light and heavy chains. In silico tryptic digestion can be performed manually or by using an online tool 'Protein Prospector' (http://prospector.ucsf.edu). The peptides generated can be screened online with protein Blast® software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The peptide sequence is compared against the target organism (biological matrix of the analyte) from an appropriate database such as UniProtKB/Swiss-prot. Any peptide scoring under 100% for either the query cover or the positive identification, represents a unique peptide and as such a potential quantifier. Thereafter, a list of potential signature peptides can be screened for amino acid stability. Amino acids such as cysteine and methionine are susceptible to oxidation leading to mass shifts of +16, +32, +48 Da depending on the number of oxidation reactions. Asparagine followed by a smaller amino acid such as glycine can readily be deamidated into aspartic acid, isoaspartic acid and succinimide during tryptic digestion, causing a mass shift of +0.98, +0.98, and -17.03 Da respectively. Also N terminal glutamine cyclization can occur at alleviated pH and prolonged digestion time. Here, the loss of ammonia leads to a mass shift of -17.03 Da [87]. Preferably, these amino acids should be avoided.

However, when stable alternatives are unavailable, these peptides can still be used if a stable isotopically labeled (SIL) internal standard is included. For example, infliximab, somatropin and nivolumab quantification with signature peptides containing a methionine were previously reported [47, 66, 68]. Also, endogenous insulin-like growth factor 1 was successfully quantified in serum with a signature peptide containing two cysteines that were protected from oxidation by attaching a carboxymethyl group on the thiol moiety through iodoacetamide alkylation and acid hydrolysis following disulfide bond reduction [88]. Nevertheless, care must be taken when these peptides are used since *in vivo* degradation of the therapeutic mAb via the above mentioned pathways can lead to an underestimation of circulating mAb as was demonstrated by Bults and colleagues in their research into deamidation of trastuzumab in human plasma [67].

After a list of potential signature peptide candidates is composed, a tryptic digest of the stock standard is preformed and run on LC-MS/MS to identify the various eluting peptides. An

example of this is illustrated in figure 4 where a number of infliximab peptides were identified using a shallow gradient (5-50% acetonitrile 0.1% formic acid in 20min). It is important to note that due to the slow scan speed of the triple quadrupole mass spectrometer a high stock standards concentration (~100µg/mL) should be digested.

Internal standard selection

Methods that include an IS are able to correct for various factors causing variability during sample analysis, such as component losses during sample preparation as well as instrument related factors such as injection, ionization and fragmentation. Depending on the IS used, different levels of corrections can be obtained (Table 2). Also in ELISA there have been attempts to incorporate IS in the assays [89]. However, the correction ability here is limited to dilution corrections only. In LC-MS/MS, SIL proteins can correct for the entire sample pretreatment and analysis because of their matching amino acid sequence and conformational folding and are considered the gold standard in quantitative proteomics [21, 90].

Table 2: Performance of various internal standards during sample workup and analysis

	Sample preparation			LC-MS/MS Analysis		
Internal Standard	Purification	Digestion	Clean-up and Enrichment	Injection	Ioniza-tion	Fragmen-tation
SIL Protein	++	++	++	++	++	++
SIL Peptide	–	–	++	++	++	++
Dimethyl Label	–	–	++	++	++	++
Flanking SIL (Extended) Peptide	–	–	++	++	++	++
Analogue Protein	++	+	+	++	+	+

SIL = Stable Isotopically Labeled
++ = Optimum correction
+ = Moderate correction
– = No correction

Unfortunately, these SIL variants of therapeutic proteins are often very expensive and only a limited number are commercially available. As an alternative, Nouri-Nigjeh and colleagues have shown that hybrid calibration, which use the therapeutic mAb of interest as 'calibrator' in combination with a SIL peptide or extended-SIL peptide as IS, can obtain accurate and precise results in whole sample digestion methods [90]. This observation was also supported by Prasad and Unadkat stating that SIL peptides can be used when maximum trypsin digestion is ensured [91]. The largest source of variability in this type of work-up originates from ionization suppression due to sample complexity. In contrast to the calibrator protein, flanking SIL peptides and extended peptides are easily dissolved in the sample matrix and due to the lack of structural folding and S-S bonds, provide easier access to the cleavage site. Therefore, the correction for digestion efficiency is expected to be very low using this approach. Furthermore, experiments performed in house using a regular SIL peptide and a SIL extended peptide showed that SIL peptide performed better than SIL extended peptide since the SIL extended peptide produced additional variability during digestion that was not correlated to variability found in calibrator protein digestion.

Dimethyl labeling was used by Ji and coworkers and was found to be a cheap way of generating multiple labeled peptides from the protein calibrator [55]. However, reaction conditions need to be carefully optimized to obtain maximum labeling efficiency. This principle has not gained a lot of ground since cheap SIL labeled peptides with high purity can easily be obtained. Analogue proteins are also frequently used to correct for sample purification and digestion (Table 1). However, the peptides generated from these analogue proteins are not identical to the signature peptides of the target mAb. Therefore, differences in charge and or hydrophobicity could lead to suboptimal correction for clean-up and enrichment steps. Moreover, differences in protein folding, solubility and disulfide bond location between the calibrator protein and the analogue may only result in moderate correction for digestion if preceding protein reduction, alkylation and denaturation was suboptimal.

Matrix effect correction for ionization relies on the elution order of the signature peptide and the IS. So, depending on the elution similarities between the signature peptide and the analogue peptide, varying levels of corrections can be achieved. This is also true for fragmentation correction, here similarities in amino acid sequences between the signature peptide and the analogue determine the levels of correction. Nevertheless, Li and colleagues have shown that when a selective purification is used, analogue proteins can perform better than SIL peptide or a SIL flanking peptide [56]. Here, sample recovery was the major contributor to the method error and therefore, by including an analogue protein that can experience the same losses as the calibrator protein, correction was achieved.

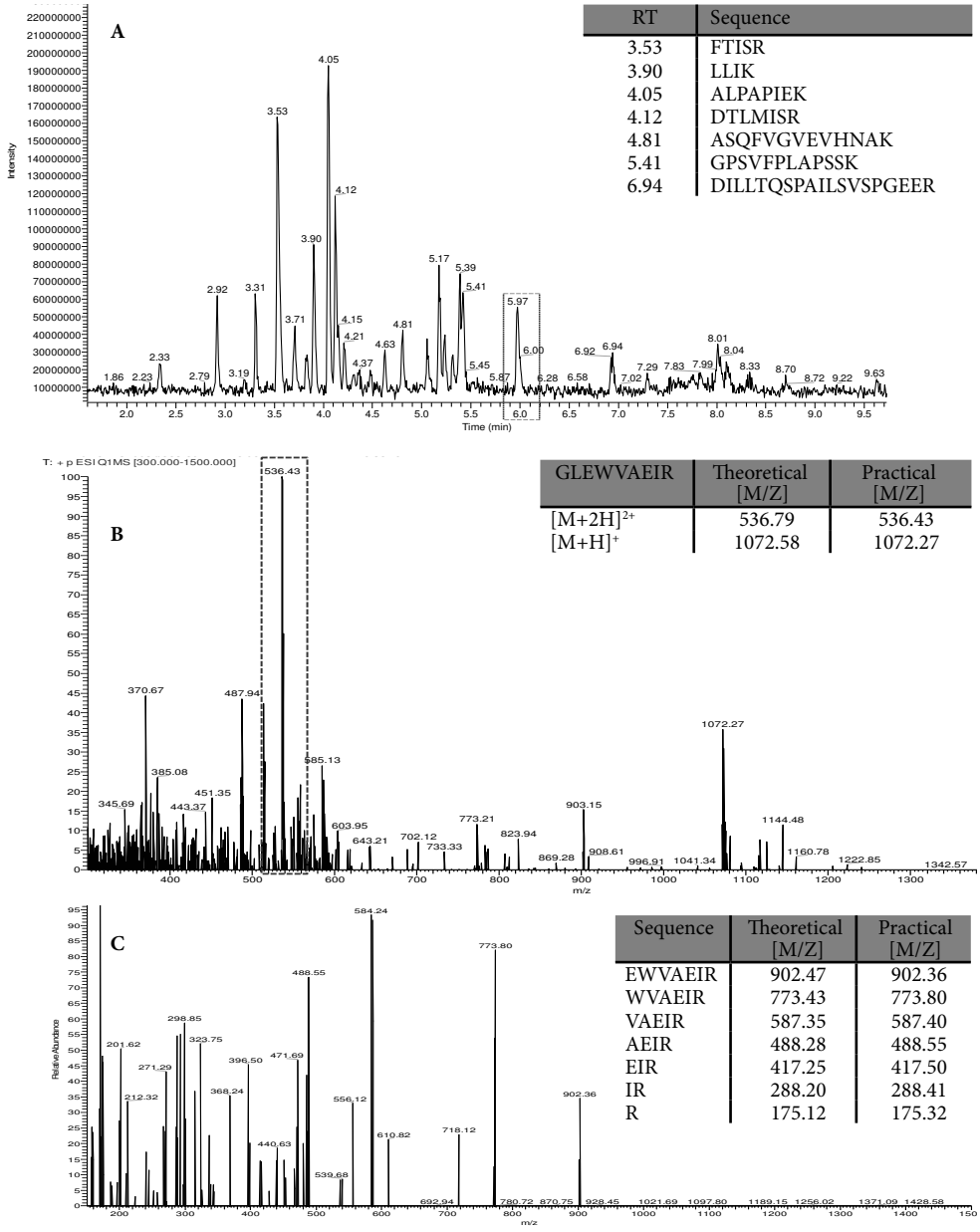
Furthermore, variability in LC-MS/MS analysis is expected to be low since reproducible ionization and fragmentations can be achieved as a result of the clean sample extract.

LC-MS/MS optimization

Chromatographic separation and MS/MS optimization are critical steps in method development and if done properly can lead to higher assay sensitivity. LC separation and MS/MS optimization are firstly performed on a digested mAb standard and after sample purification and digestion conditions are optimized, LC separation is re-evaluated with a mAb spiked sample of the biological matrix of interest. When sample purification methods are used that result in clean extracts, such as targeted sample purification, short LC run times (<5 min) can successfully be achieved [34, 48, 56, 62, 72]. Nevertheless, columns with higher plate numbers and longer gradient times might be required to separate isobaric interferences and matrix effects when generic sample purification methods are used.

High resolution instruments such as the Orbitrap, time of flight (TOF) or ion trap (QTap) can also be used and may provide the necessary selectivity. However, matrix effects can only be eliminated through sample cleanup and sufficient LC separation. Detection limits can be lowered by selecting the optimal signature peptide but also by monitoring the most abundant precursor and product-ions. Signature peptides with chain lengths of around 20 amino acids produce precursors consisting of single, double, triple and quadruple charged states. Therefore, a precursor mass-scan needs to be performed to determine the most abundant charged state for quantification (Fig. 4B). Consecutively, the most intense product-ion can be found by performing a mass-scan after collision-induced dissociation (CID) of the most abundant precursor peak (Fig. 4C).

In contrast to triple quadrupole MS, HRMS instrumentations have a higher full mass scan rate of >12 Hz and can easily obtain precursor and product-ion scans of the desired signature peptide with high accuracy and sensitivity using low amount of sample [92]. This combination of most abundant precursor and subsequent product-ion is termed selected reaction monitoring (SRM) and can further be optimized for collision gas pressure and collision energy. This can easily be done through infusion of a synthesized peptide directly into the MS or alternatively, can be optimized by performing multiple injections of a digested protein, each time with a different collision setting. Unfortunately, different MS instrumentation can generate different precursor charge ratios and product ion profiles. Therefore, SRM settings are not interchangeable between different instruments. This is demonstrated in figure 4C and figure 5 where GLEWVAEIR fragments were scanned by 2 different MS instruments. In figure 4C, the CID with a triple quadrupole, fragment 773.80 was higher than fragment 488.55, while this was the opposite with higher energy collision dissociation (HCD) with the orbitrap (Fig. 5).



RT	Sequence
3.53	FTISR
3.90	LLIK
4.05	ALPAPIEK
4.12	DTLMISR
4.81	ASQFVGVEVHNAK
5.41	GPSVFPLAPSSK
6.94	DILLTQSPAILSVSPGEER

GLEWVAEIR	Theoretical [M/Z]	Practical [M/Z]
$[M+2H]^{2+}$	536.79	536.43
$[M+H]^{+}$	1072.58	1072.27

Sequence	Theoretical [M/Z]	Practical [M/Z]
EWVAEIR	902.47	902.36
WVAEIR	773.43	773.80
VAEIR	587.35	587.40
AEIR	488.28	488.55
EIR	417.25	417.50
IR	288.20	288.41
R	175.12	175.32

Figure 4: Signature peptides identification of infliximab tryptic digest with a triple quadrupole operating in full scan MS (300-1500 m/z) (A), GLEWVAEIR precursor elucidation though comparison with theoretical mass (B) and finally, conformation of the precursor sequence through fragmentation (20 eV) of [M+2H]2+ and product ion scan (150-1500m/z) (C).

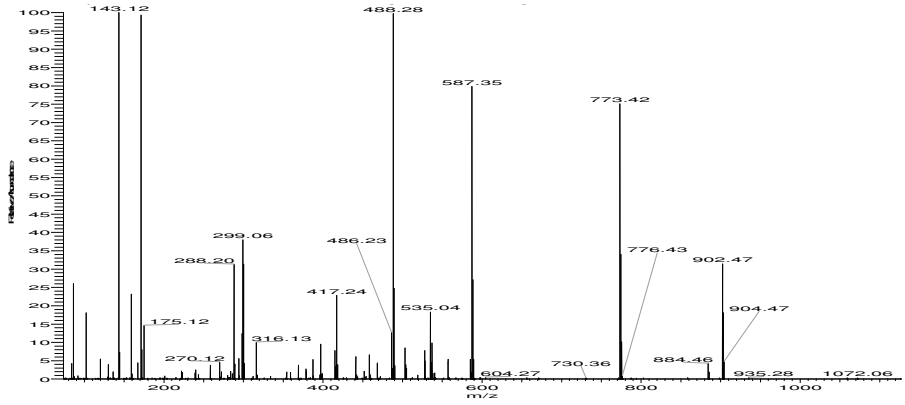


Figure 5: Product ion scan of GLEWVAEIR peptide with QExactive Orbitrap

Nevertheless, three most intense product ions per signature peptide should be monitored and only after complete validation, the best performing signature peptide with its concomitant product ion can be selected as quantifier. Other signature peptides that have successfully passed validation can be selected as qualifiers and can be used for quality assurance. Here, sample results from the quantifier peptide can be compared with those of the qualifier peptide during routine analysis. Large variations (>15%) between results could be caused by isobaric interferences which can be present in one signature peptide but not necessarily the other and would require further investigation. However, for each signature peptide a SIL internal standard equivalent is required for quantification this would lead to increased costs when SIL peptides are used.

Sample purification

Arguably, the most laborious step in the development of a successful bioanalytical method is sample purification. Sample purification is necessary to eliminate interfering proteins and reduce sample complexity (Table 3) and over the last decades a myriad of strategies have been reported. Target-specific sample purification in a human bio-matrix utilizes an anti-idiotypic antibody or ligand fixed to a solid support such as a magnetic bead or 96 well plate (Fig 6A). Here, only the active therapeutic mAb fraction with at least one free epitope can be purified. This principle was used by our group to purify active infliximab in human serum with its antigen tumor necrosis factor alpha bound to a 96-well plate by means of biotin – streptavidin interaction [72]. A similar principle was reported for the purification of bimagrumab in

human serum using activin receptor type 2B which was cross-liked to sepharose magnetic beads by means of NHS (N-hydroxysuccinimide) reagent [77].

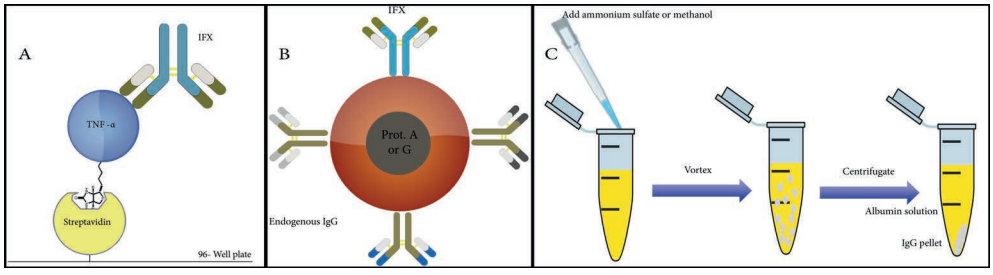


Figure 6: Sample purification methods using; TNF alpha to selectively capture infliximab (A), Protein A or G to capture FC portion of the antibodies (B) and IgG pellet precipitation with ammoniumsulfate or methanol (C). Fig 6A was reprinted from Journal of Chromatography A, 1454, M. El Amrani, M.P. van den Broek, C. Gobel, E.M. van Maarseveen, Quantification of active infliximab in human serum with liquid chromatography–tandem mass spectrometry using a tumor necrosis factor alpha-based pre-analytical sample purification and a stable isotopic labeled infliximab bio-similar as internal standard: A target-based, sensitive and cost-effective method, 42-48. Copyright (2016), with permission from Elsevier [72].

The use of anti-idiotypic antibodies, which bind the complementarity-determining regions (CDRs) of the therapeutic mAb, is exemplified with the purification of trastuzumab in human serum with anti-trastuzumab idiotypic antibodies [42]. Purifications with anti-human Fc antibodies in animal bio-matrices can also be considered 'targeted'. Here, only the human/humanized therapeutic antibody will be captured, resulting in the quantification of total therapeutic antibody [48, 56, 59]. When combining a targeted sample pre-treatment with LC-MS/MS measurement, low detection levels (< 0.5 mg/L) can be achieved through background noise reduction (Table 3). In contrast, a generic sample work-up aimed to capture the entire IgG fraction in serum can be used. Protein A and G are bacterial cell wall proteins that bind IgG via their Fc region, thus protecting it in vivo from the immune system. These proteins achieve relatively clean extracts and have been used with success to purify the total therapeutic mAb (Fig. 6B) [45, 53, 65, 68-71, 76, 80].

Experiments performed in-house and others have shown that the ammonium sulfate (AS) precipitation method is highly efficient in the removal of albumin fraction, which comprises of around 60% of total plasma protein (Fig. 6C) [85, 94]. Proteins with low solubility (usually large proteins), are precipitated first at increasing AS concentration, leaving the highly soluble, smaller proteins and molecules in solution [95]. This is a cost effective and fast way to remove unwanted proteins and was successfully employed for dinutuximab and infliximab quantification in human serum with LC-MS/MS [66, 85]. The major advantage of AS versus the widely used MeOH pellet digestion method (Table 1), is that the protein pellet retains its

tertiary conformation allowing for fast re-solvation for reduction and alkylation [95]. Each of the above-mentioned methods has its merits and challenges. The targeted assay tends to be more time consuming and requires ligands or antibodies which can be scarce and expensive. However, the targeted assay provides lower detection limits because of clean extracts and measures the 'active and free' mAb fraction which are likely associated with loss of therapeutic response [72]. In contrast, generic sample purification methods are easy and fast to perform and measure total mAb fraction.

Table 3: Estimated and theoretical levels of purifications of human plasma proteins using different sample preparation strategies. Theoretical data for protein A and G purification obtained from Thermo Scientific [93].

Plasma Components	Plasma Conc. [g/L]	Targeted Purification	Protein A	Protein G	AS* Precipitation	MeOH** Pellet digestion
Albumin (60 kDa)	45	++	++	++	++	+
IgG (150 kDa)	10	++	–	–	–	–
Fibrinogen (340 kDA)	2.5	++	++	++	–	–
Transferrin (80 kDa)	2.5	++	++	++	++	+
IgA (320 kDa)	2	++	+	++	–	–
Alpha-1 Anti-Trypsin (54 kDa)	1.5	++	++	++	++	+
Phospholipids (<1 kDa)	1	++	++	++	++	++
IgM (900 kDa)	1	++	+	++	–	–
IgD (180 kDa)	0.02	++	++	++	–	–
IgE (200kDa)	0.0002	++	++	++	–	–

*AS = Ammonium Sulfate
**MEOH = Methanol
++ = Efficiently eliminated
+ = Moderately eliminated
– = Not eliminated

Importantly, total and free fraction results can show poor correlation, especially when anti-drug-antibodies (ADA) are present in the sample [21, 96]. Therefore, correlation studies must be performed when switching from one assay to another. Nevertheless, there are numerous reports of good agreements between free fraction measured by ELISA and total fraction measured by LC-MS/MS [48, 56, 60, 78, 97], even in subsets consisting of ADA positive samples, as was demonstrated by Willrich and colleagues in the quantification of infliximab in human serum [66].

Digestion conditions

After sample purification, the therapeutic mAb needs to be denatured (unfolded) to allow, in the subsequent step, the digestive enzyme easier access to the cleavage sites. The protein tertiary structure is maintained by hydrophobic, ionic, hydrogen and disulfide bonds. Thus, abrogation of these interactions and bonds can achieve faster and efficient digestion. Disulfide bonds can be reduced with 5 mM DTT or tris (2-carboxyethyl)phosphine (TCEP). TCEP is a stronger and more stable reducing agent compared to DDT. However, DTT is most frequently used due to its neutral pH being more compatible with downstream trypsin digestion. Usually, a reduction is carried out under heating conditions (around 60 °C) to speed up the reaction process and to aid in protein denaturation. Urea concentrations >6 M can also unfold the protein structures, but sample dilution or dialysis is then required to lower the urea concentration <1 M prior to trypsin digestion. Furthermore, undesirable physiochemical reactions can occur when using urea at elevated temperatures. Recent publications focus mainly on unfolding the protein via heating >70 °C with or without MS compatible surfactants such as RapiGest™ [73, 76, 78]. The use of sodium dodecyl sulfate (SDS) surfactant has also been reported [50, 85]. However, when SDS is left in the buffer solution prior to trypsin digestion, the proteolytic enzyme would denature and subsequent MS analysis would suffer from ionization suppression. As proteolytic enzyme, trypsin is mostly preferred for bottom up proteomics, because it cleaves the peptide bonds following arginine (R) and lysine (K), two basic amino acids that are easily ionized during electrospray ionization. Trypsin is active in a buffered solution with low ionic strength <0.1 M with pH 7 – 9 [72].

Digestion efficiency is dependent on factors such as trypsin to protein ratio, temperature, time, protein accessibility and the presence of trypsin inhibitors such as alpha-1 anti-trypsin [50]. Digestion efficiency can further be improved by incorporating methylated trypsin which can retain its activity during digestion and can thus be used in lower amounts [98] or by addition 1mM calcium ions to the solution which aids in trypsin stability [99]. Treatment with 6-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) is also required to disable chymotrypsin activity in native trypsin [100], since untreated chymotrypsin would cleave the protein at different locations (tyrosine, tryptophan and phenylalanine) affecting signature peptide recoveries.

Immobilized trypsin is increasingly being implemented, since it offers fast and complete digestion and can be used in combination with high temperatures [68, 71, 78, 101, 102]. Immobilization secures trypsin on a silica bead or agarose resin and retains its active conformation during high temperature sample denaturation which is necessary for fast protein digestion. Moreover, efficient digestion is promoted by immobilizing excess mounts of trypsin thus favourable enzyme to protein ratio is achieved. Combinations of enzymes

can also be incorporated to improve digestion efficiency such as the inclusion of Enzyme LysC, which tolerates high urea concentrations, in combination with trypsin [33]. Since these combination of enzymes, immobilized trypsin and methylated trypsin are significantly more expensive than TPCK trypsin it is recommended to test these enzymes side by side to determine their added value.

Method validation

After optimization of assay conditions the method is subjected to validation following FDA or EMA guidelines for bioanalytical method validation [36, 103]. Parameters, such as LLOQ, accuracy, precision, matrix effect, linearity, stability and carry-over should comply with requirements as states in these guidelines. Furthermore, it is strongly recommended to carry out a cross-validation against an established method to determine whether the methods are strongly correlated. In regards to the latter, it should be stressed that measurement of different therapeutic mAb fractions, e.g. free or total, can result in inter-assay differences. Moreover, inter-assay variation can occur, even when the same fraction is measured. This is exemplified by the comparison of active infliximab quantification in human serum with LC-MS/MS versus ELISA-based assay [72]. Here, the sandwich type ELISA required two free mAb paratopes, one paratope for fixation and the other for detection, while the LC-MS/MS assay only required one free paratope. This meant that the infliximab fraction with only one free paratope could not be quantified with the ELISA assay which may have resulted in an underestimation of free infliximab in serum.

Conclusion and discussion

An overview of state-of-the-art LC-MS/MS methods used for quantification of therapeutic mAbs in biomatrices is provided. Current literature on peptide level quantification is summarized in six workflow steps, and benefits and drawbacks in each step of method development have been critically evaluated. We conclude that LC-MS/MS instruments offer fast method development and multiplexing capabilities and will continue to replace ligand-binding assays as these instruments get cheaper, improve in terms of sensitivity and mass accuracy with each generation. Innovations and improvements in materials, such as the immobilization of trypsin and magnetic beads conjugated with various ligands, will aid in speeding analysis times while providing high recoveries and sensitivities. Also, with increasing availability of stable isotopically labeled mAbs, method robustness, precision and

accuracy will further improve.
Top-down and middle-down quantitative proteomics are expected to become more important as newly developed mAb are mostly fully human or humanized. Improvement to instrument hardware and software are needed to facilitate the growth in this area. As more awareness in the scientific community is growing to the possibilities that these methods have to offer, LC-MS/MS methods have the potential to become the technique of choice for mAb quantification in preclinical and clinical settings.

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2.2

Quantification of active infliximab in human serum with liquid chromatography–tandem mass spectrometry using a tumor necrosis factor alpha-based pre-analytical sample purification and a stable isotopic labeled infliximab bio-similar as internal standard: A target-based, sensitive and cost-effective method.

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Abstract

The therapeutic monoclonal antibody Infliximab (IFX) is a widely used drug for the treatment of several inflammatory autoimmune diseases. However, approximately 10% of patients develop anti-infliximab antibodies (ATIs) rendering the treatment ineffective. Early detection of underexposure to unbound IFX would result in a timely switch of therapy which could aid in the treatment of this disease.

Streptavidin coated 96 well plates were used to capture biotinylated-tumor necrosis factor-alpha (b-TNF-α), which in turn was used to selectively extract the active form of IFX in human serum. After elution, IFX was digested using trypsin and one signature peptide was selected for subsequent analysis on liquid chromatography-tandem mass spectrometry (LC-MS/MS). The internal standard used was a stable isotopic labeled IFX bio-similar.

The assay was successfully validated according to European Medicines Agency (EMA) guidelines and was found to be linear in a range of 0.5 – 20 μg/mL (r2=0.994). Lower limit of quantification for the assay (<20% CV) was 0.5 μg/mL, requiring only 2 μL of sample. Cross-validation against enzyme-linked immunosorbent assay (ELISA) resulted in a high correlation between methods (r2=0.95 with a ρc=0.83) and the accuracy was in line with previously published results.

In conclusion, a sensitive, robust and cost-effective method was developed for the bio-analysis of IFX with LC-MS/MS by means of a target-based pre-analytical sample purification. Moreover, low volume and costs of consumables per sample promote its feasibility in (pre) clinical studies and in therapeutic drug monitoring. This method should be considered as first choice due to its accuracy and multiple degree of selectivity.

Introduction

Infliximab (IFX), an immunoglobulin G (IgG) based tumor necrosis factor alpha (TNF-α) blocking antibody, has been approved by the EMA and the FDA for the treatment of several autoimmune diseases, such as Crohn's disease, ulcerative colitis, ankylosing spondylitis and rheumatoid arthritis to induce and retain remission [1, 2]. One year after initiation of IFX therapy, 10% of patients lose response to the treatment [3]. It is speculated that this is largely due to induction of immunogenicity where patients develop anti-infliximab antibodies (ATIs) that bind to IFX rendering it ineffective. Therapeutic drug monitoring of the biological active fraction of IFX in patients' serum has shown great promise to improve treatment outcomes in patients suffering from inflammatory autoimmune diseases [4-7].

The traditional way of analyzing monoclonal antibodies such as IFX is by ELISA. However, there are some noteworthy differences in sensitivity and selectivity attained with various IFX ELISA assays [8, 9]. These differences are mainly attributed to the assay design for capturing and detecting the monoclonal antibody of interest. Furthermore, ELISA assays are vulnerable to cross reactivity, this is when the detecting antibody lacks specificity and attaches itself to a soluble receptors or to endogenous IgG in serum instead of the therapeutic antibody of interest [8, 10]. Moreover, ELISA methods lack the linear dynamic range and due to the higher risk of cross reactivity requires an extensive validation [11].

It has been demonstrated that LC-MS/MS preceded by sample purification and protein digestion can provide the high sensitivity and specificity required for the quantification of monoclonal antibodies in serum [12]. Sample purification prior to MS analysis is necessary to remove serum proteins and salts that otherwise would interfere with the analysis. There have been various methods published dealing with sample purification for therapeutic antibodies in serum. Methods such as pellet digestion remove albumin from serum in the supernatant layer by using organic solvents or salts, here the pellet retains all IgGs including the therapeutic antibody [12-15]. Albumin accounts for approximately 50% of total serum proteins, thus the removal of albumin from serum enhances the sensitivity of the method [16]. Other approaches focus on capturing IgGs in serum by means of immuno-affinity purification using either protein A, protein G, or anti human Fc antibodies attached onto a solid support such as magnetic beads [17-23]. Thereafter, the therapeutic antibody is eluted and digested using trypsin and finally one 'signature' peptide (a non-endogenous peptide) is selected for quantification. However, these sample purification methods mentioned above are nonspecific for the active form of the therapeutic antibody. Furthermore, immunoglobulins (Ig's) are amongst the most abundant proteins in serum [24, 25]. These "naturally present" Ig's are extracted together with the therapeutic antibody during these types of sample purifications and they pose a potential source of chromatographic interference and ionization suppression

during MS analysis. In addition, high levels of Ig's will compete with the therapeutic antibody for the binding sites during these types of immuno-affinity interaction, potentially harming recovery rates.
Indeed, a selective sample preparation would be able to eliminate or reduce the drawbacks found in the generic sample purification approaches described above. Therefore, in this manuscript we propose a selective purification of active IFX (infliximab with an unbound paratope) in human serum by means of its antigen TNF-α, followed by trypsin digestion and LC-MS/MS analysis. The proposed purification is based on immunoaffinity using biotinylated TNF-α (b-TNF-α) which has been fixed onto a streptavidin coated 96 well plate.
The benefit here are, cleaner extracts due to the use of selective interactions, which would lead to a reduction in the noise level and a lowering of the detection limit. Furthermore, due to the selective nature of the test, only the antibody of interest (IFX) is captured and eluted this would require a minimal amount of the capturing antigen (b-TNF-α). Moreover, cleaner extracts would require a minimal amount of trypsin for the subsequent digestion. Also, due to the coupling of TNF-α onto a 96 well plate instead of beads, higher throughput, ease of use and faster sample preparation times are facilitated. These factors would reduce the cost of the test significantly and most importantly the use of a stable isotope labelled IFX bio-similar as internal standard (IS) would aid in obtaining accurate and precise results. This is due to the possibility of introducing the IS at the beginning of the sample preparation, thus providing ideal correction for loses occurred during sample pre-treatment, enzymatic digestion and LC-MS/MS analysis.

Materials and methods

Chemicals and reagents

Infliximab (Remicade™), was obtained from Janssen Biologics B.V. (Leiden, The Netherlands) as lyophilized powder and was reconstituted in distilled water to a final concentration of 10 µg/µL, 100 µL aliquots of this solution were pipetted in Eppendorf LoBind™ Microcentrifuge tubes and stored at -80 °C . Stable isotope Internal standard infliximab bio-similar was obtained from Promise advanced proteomics (Grenoble, France) as a 10 µg/10 µl solution. Biotinylated human recombinant TNF-α was obtained from ACRO biosystems (Newark, DE, USA) and dissolved in Phosohate Buffered Saline (PBS), 0.1 % Tween-20, 1 % BSA to 50 µg/mL. Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Fisher (Waltham, MA, USA). MS grade modified trypsin was obtained from Promega

(Madison, WI, USA) and was dissolved to 1 µg/µL in 50 mM acetic acid and aliquoted in the Eppendorf LoBind™ Microcentrifuge tubes. Aliquots were stored at -80 °C. Drug-free human serum was obtained from BIO-RAD (Irvine, CA, USA). All other reagents and LC-MS grade mobile phase solvents were obtained from Sigma (Saint Louis, MO, USA).

Preparation of standards, internal standard and QCs

The working IFX solution (20 µg/mL) was prepared from the 100 µL aliquot (10 µg/µL) by adding 900 µL drug free human serum (DFHS) to obtain a concentration of 1 µg/µL. This solution was diluted further to 20 µg/mL in DFHS. Aliquots were stored in LoBind™ eppendorf tubes at -80 °C. Standards at concentrations of 0.5, 1, 2.5, 5, 10, 20 µg/mL were prepared fresh from the working solution by dilution in DFHS. The 10 µg/10 µl IS solution was diluted to 25 µg/mL. Aliquotes were stored at -80 °C The working IS solution of (2.5 µg/mL) was prepared from the stock solution (25 µg/mL) by dilution in DFHS. Quality Control samples (QCs) at lower limit of quantification (LLOQ) (0.5 µg/mL) , QC low (1.5 µg/mL), QC med (6 µg/mL) and QC high (15 µg/mL), were prepared in DFHS from a different stock solution to that used to make the standards. Aliquots were stored at -80 °C.

Instrumentation and chromatographic conditions

Sample purification was performed on a vibramax 100 plate shaker, Heidolph Instruments (Schwabach, Germany). Sample drying was performed on a HETOVAC™, VR-1 (Allerød, Denmark). All experiments were performed on an Ultimate 3000 UHPLC Dionex (Sunnyvale, CA,USA) coupled to a TSQ Quantiva, Thermo Fisher (Waltham, MA, USA). The analytical column was Acquity UPLC™, BEH 2.1 x 50 mm, 1.7 µm particle size, Waters (Milford, MA, USA) and was maintained at 50 °C. The mobile phases were: (a) 0.1 % formic acid in water; (b) 0.1 % formic acid in ACN. The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/10 (% B), 3/35, 3.01/100, 4.25/100, 4.26/10 and 5.5/10. The flow rate was 0.5ml/min and the run time was 5.5 min. The MS was operated in positive mode with spray voltage of 3.5 kV, Ion Transfer Tube Temperature 350 °C, vaporizer temperature 400 °C, aux gas pressure 15 Arb, sheath gas pressure 50 Arb, sweep gas pressure 0 Arb and collision gas pressure 2.5 mTorr. The precursor ions, product ions, collision energy and radio frequency (RF) lens settings are listed in Table 1 for IFX and for the internal standard.

Table 1. TSQ Quantiva Mass Spectrometry Conditions for SRM transitions for the signature peptide liberated from IFX variable light chain after digestion with trypsin and the internal standard stable isotopic labelled IFX bio-similar.

Peptide sequence	Precursor (m/z)	Product (m/z)	Product Ion type	CE[a] (V)	RF[b] (V)
DILLTQSPAILSVSPGER (Quantifier ion)	948.53	545.25	y5	25	110
DILLTQSPAILSVSPGER (Qualifier ion)	948.53	731.37	y7	25	110
DILLTQSPAILSVSPGER (Qualifier ion)	948.53	1125.63	y11	25	110
DILLTQSPAILSVSPGER[13C6,15N4], (IS)c	953.53	555.25	y5	25	110

[a] CE: Collision energy

[b] RF: Radio frequency lens

[c] IS: Internal standard

Sample preparation for LC-MS/MS analysis

Sample preparation was based on immunoaffinity where active IFX was bound to b-TNF-α, which in turn was bounded to streptavidin coated 96 well plate (Fig. 1). B-TNF-α was coupled to a streptavidin coated 96 well plate by pipetting 5 µL b-TNF-α (50 µg/mL) and 195 µL PBS (0.1% Tween-20) in each well, followed by overnight mixing on a plate shaker (300 rpm) at room temperature. The plate were washed 3 times with 200 µL PBS (0.1% Tween-20). Then, in a LoBind™ eppendorf tube, 200 µL PBS (0.1% Tween-20) was added, followed by 2 µL (standard, QC or serum sample) and 4µL IS (2.5 µg/mL). This solution was mixed (gently) and transferred to the b-TNF-α pre-treated 96 well plate and allowed to bind for 1 hour at room temperature on a plate shaker (300 rpm). Then, the wells were washed 3 times with 200 µL PBS (0.1 % Tween-20), followed by 5 times water wash to remove salts and tween.

Bound IFX was extracted by adding 100 µL elution solution (48.5% MEOH : 48.5% H2O : 3% formic acid) and vortex mixed for 10 minutes at 900 rpm on a plate shaker. 100 µL extracts were transferred to a 2 mL glass vials and dried for 1 hour at 45 °C on the HETOVAC™. The samples, QC's and standards were reconstituted in 150 µL Tris (50mM, pH 9.5). Then, 5 µL DTT 100mM was added, mixed for 5 minutes at 1500 RPM and allowed to react on a block heater set at 60 °C for 30min. Samples were briefly spun down. Then, 5 µL trypsin (0.05 µg/µL) dissolved in acetic acid 2.5mM was added and gently mixed. Then, the tubes were placed in an oven set at 37 °C for overnight digestion. Finally, trypsin activity was stopped through the addition of 30 µL formic acid 10 % in methanol and 25 µL was injected and analyzed on LC-MS/MS.

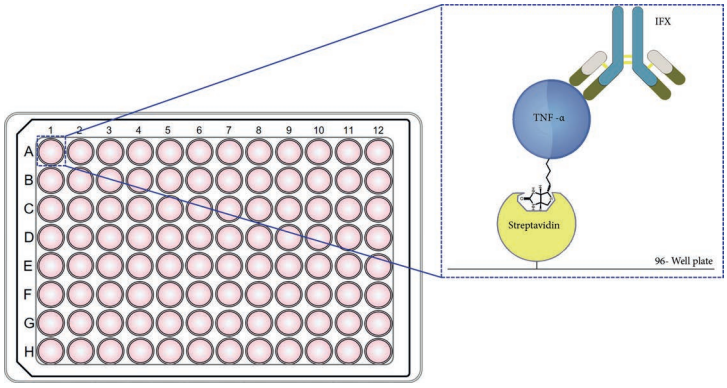


Fig. 1. Principle of immunoaffinity purification of IFX using biotinylated TNF-α and streptavidin-coated 96 well plate

ELISA method for IFX quantification

The ELISA reference method, used in this study to evaluate LC-MS/MS IFX results, was developed by Sanquin and validated according to ICH guidelines [26]. The steps involved are indicative of sandwich type ELISA. In brief, the procedure is based on capturing active IFX with TNF-α which was bound to a monoclonal antibody on a polystyrene microtiter well plate. After washing the unbound serum components, biotinylated anti-IFX antibody was added. After washing, horseradish peroxidase (HRP) conjugated with streptavidin was added. Then, excess HRP was washed off followed by the addition of the substrate solution. HRP converts the substrate solution to a colored product in proportion to the amount of IFX present. Finally, a stop solution is added to terminate the reaction and the absorbance was measured in a microtiter plate reader.

Sample selection for comparative study

Routine Therapeutic Drug Monitoring samples were collected from patients that were treated with IFX. A portion of the sample was send for ELISA analysis at Sanquin (Amsterdam, The Netherlands) and the remainder was stored at -80 °C for LC-MS/MS analysis. The samples for the comparative study were selected based on their therapeutic range and on the presence of anti-IFX antibodies. The study was carried out on 24 samples from a concentration range of 0 to 20 µg/mL, of which 7 samples contained anti-IFX antibodies. All serum samples used in this study, were collected with patient's consent.

Validation of infliximab LC-MS/MS method

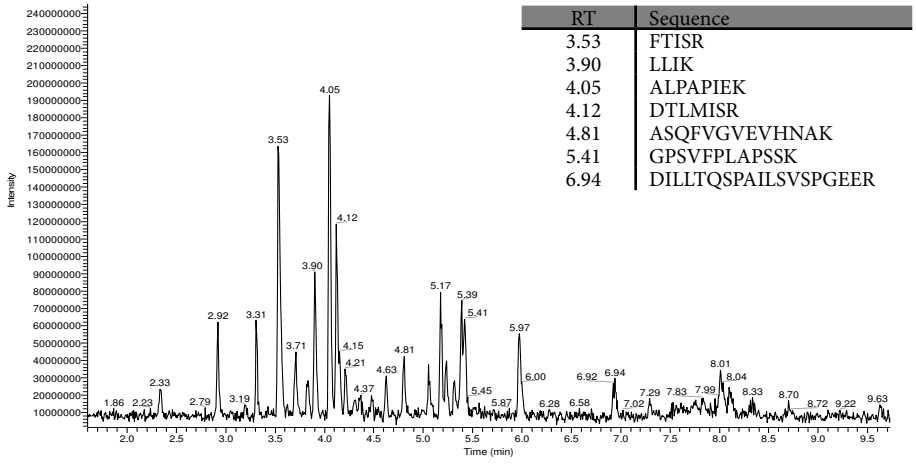
A linear calibration curve was evaluated on three separate days and was established consisting of six standards ranging from 0.5 to 20 µg/mL. LLOQ was determined by calculating the ratio of LLOQ signal to the blank (DFHS) spiked with IS signal on three different days in 5 folds. Selectivity was tested using 6 blank human serum samples. Specificity was tested using spiked Adalimumab, Etanercept, Azathioprine and Cetuximab at 10 µg/mL in serum. Within-run and between-run accuracy and precision, which is a measure for repeatability and reproducibility of the analytical method, was validated by analyzing LLOQ, QC low, QC med and QC high in five folds during 3 days. The data obtained for each concentration level was evaluated with single factor ANOVA. Accuracy was expressed as percentage bias. Precision was expressed as percent coefficient of variation (% CV) and was calculated from the ANOVA derived mean squares (MS). Matrix effects of both IFX and IS were investigated by performing sample purifications on 6 randomly chosen blank human serum samples in duplicate, after washing and eluting, one set of extracts were spiked at QC low (1.5 µg/mL) and the other set were spiked at QC high (15 µg/mL) level. Clean extraction solutions were also spiked at QC low and QC high and were used as the matrix free reference. Then 4 µL IS (2.5 µg/mL) was added and samples were treated according to the procedure described above continuing on from drying step of 1 hour at 45 °C. The IS-normalized matrix factor was calculated for each blank human serum sample by dividing the matrix factor (MF) of IFX (peak area IFX in the presence of matrix divided by peak area IFX in absence of matrix) by the MF of the IS. The matrix effect was expressed as % CV of the IS-normalized MF. Although lyophilized IFX can handle temperature extremes of up to 30 °C for a period of 12 months [27], the stability of IFX in serum is unknown. Furthermore, the effects of IFX denaturation due to sample handling (which could lead to IFX deactivation) could also reduce the storage period of the sample. Therefore, IFX stability was tested according to EMA guidelines at QC low and QC high level during 2 freeze and thaw cycles and the percentage bias was calculated.

Results and discussion

Method development

The 144 kDa molecular mass of infliximab exceeds the typical mass range of a triple quadrupole mass spectrometer. Therefore, protein digestion plays an integral part in LC-MS/MS analysis of biopharmaceutics. We have opted for using trypsin to produce smaller peptides, because

peptides liberated this way contain a terminal lysine or arginine, two basic amino acids that are easily ionized during electrospray ionization. During IFX digestion, a multitude of peptides were produced (Fig. 2). A signature peptide was selected based on four criteria; number (n) of amino acids in the peptide chain 6<n <20, the signal intensity, absence from the human genome (search performed on pBLAST™ using the human database swissprot) and stability of the comprising amino acids. After careful consideration, D[1]ILLTQSPAILSVSPGER peptide was selected as the signature peptide. This is a terminal peptide located in the variable light (VL1) portion of IFX which can be easily liberated. The peptides G[44]LEWVAEIR from the variable heavy chain (VH44) and G[124]PSVFPLAPSSK from the constant heavy chain (CH124) were monitored at 536.8 → 587.4 and at 593.8 → 699.4 m/z respectively solely to optimize the buffer type and strength for tryptic digestion. Stable isotopic labeled infliximab bio-similar, containing labeled arginine (13C6; 15N4) and lysine (13C6; 15N2) was chosen as the internal standard and was spiked at 5 µg/mL onto 2 µL samples, QC's and standards, thus requiring only 10 ng labeled IS. The SRM transitions of the precursors and the product ions were selected based on the ones that provided the lowest interference and the highest signal intensity. These transitions were found during MS optimization of collision energy settings (data not shown).



RT	Sequence
3.53	FTISR
3.90	LLIK
4.05	ALPAPIEK
4.12	DTLMISR
4.81	ASQFVGVEVHNAK
5.41	GPSVFPLAPSSK
6.94	DILLTQSPAILSVSPGEER

Fig. 2. Mass spectrometry scan (300-1500 m/z) of precursor peptides liberated from IFX stock 100 µg; Disulfide bond reduction performed with DTT (5 mM) at 60 °C for 1 hour, followed by digestion with trypsin (5 µg) at 37 °C for 4 hours. Identification based on precursor mass and fragmentation pattern (data not shown). Column used BEH (Waters), 1.7 µm, 150x2mm, C18, gradient used: 0 (min)/ 5 (% B), 19/50, 19.1/100, 21.0/100, 21.1/5, 25/5. Mobile phase: A= 0.1% formic acid in water and B= 0.1% formic acid in Acetonitrile.

Immunoaffinity sample purification was based on the bait and prey principal. Here 0.25µg b-TNF-α was captured on top of streptavidin coated 96 well plate (Fig. 1) which in turn was used to capture biologically active IFX fraction in serum (IFX with at least one free paratope). 0.25 µg b-TNF-α corresponds to 9.6 ρmol protein which falls within the manufacturer stated threshold of ~125 ρmol biotinylated protein per streptavidin coated well. Furthermore, compared to the highest standard (0.04 µg or 0.27 ρmol IFX), 0.25 µg b-TNF-α provides 18× molar excess to available IFX binding sites which is highly desirable for a fast extraction. The 96 well plates accommodated a high sample throughput, ease of use and a fast sample preparation time. The method was validated according to the latest EMA guidelines Rev. 1, with coming into effect date of 1 February 2012.

Streptavidin coated 96 well plate capacity test

Streptavidin has been reported to show a strong binding affinity towards biotin [28]. Furthermore, due to its near neutral isoelectric point, it possesses low non-specific binding. Based on these characteristics, b-TNF-α in conjunction streptavidin high binding capacity coated 96 well plates were chosen for the immunoaffinity purification. The capacity of these plates, which define the linear working range of the analytical method, was determined by the following test. The test was performed using the sample preparation procedure described above with some modifications, namely, the standard range was extended to 0.1-0.5-1-5-10-20-30-40-50-60-70-80-90 µg/mL and the internal standard was added after elution in this experiment, in order to correct solely for the losses observed after elution.

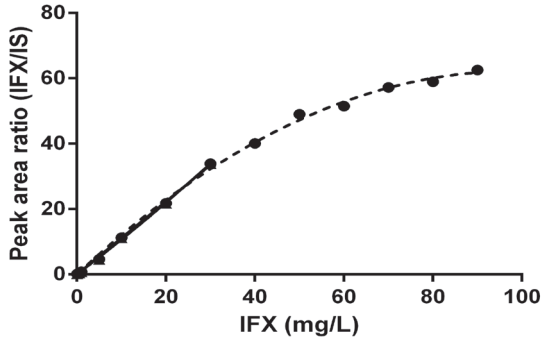


Fig. 3. Streptavidin high binding capacity coated 96 well plate tested by allowing 250 ng or 14.4 pmol b-TNF-α and 2 µL standard (0.1 - 90 µg/mL) to bind, followed by addition of IS after elution (4 µL, 2.5 µg/mL), drying, trypsin digestion and LC-MS/MS quantification.

As illustrated by figure 3, the calibration line starts to bend around 30 µg/mL IFX, which corresponds to 0.06 µg IFX per well. The therapeutic trough level range for patients treated with IFX lies between 3 and 7 µg/mL, therefore we have chosen to limit our calibration range to the upper level of 20 µg/mL for therapeutic drug monitoring purposes. One hour binding time was selected to reduce the analysis time, however, an extended calibration range might be achieved by increasing the binding time.

Optimizing buffer type and strength for tryptic digestion

In this experiment we examined the buffer type and strength that yield the highest signals for three peptides (VL1, VH44 and CH124). The use of ammonium bicarbonate (ABC) and tris (hydroxymethyl) aminomethane (Tris) buffers both ranging from 50 to 200 mM buffer strength was explored. The buffers were adjusted with HCl to pH 8 for both buffer types at all buffer strengths. The test was performed in triplicate, 10 µL IFX working solution was diluted in multiple vials with 90 µL Tris or ABC at various buffer strengths, ranging from 50 to 200 mM with 50 mM increments. DTT was freshly made and added to the test solutions to obtain a final concentration of 2 mM. The vials were heated to 60 °C for 60 min to reduce the disulfide bonds. After the vials were cooled to room temperature, 10 µL trypsin (0.01 µg/µL) was added and digested for 3 hours at 37 °C. Finally, 10 µL formic acid (10% in methanol) was added to stop the reaction and 25 µL was injected and analyzed on the LC-MS/MS.

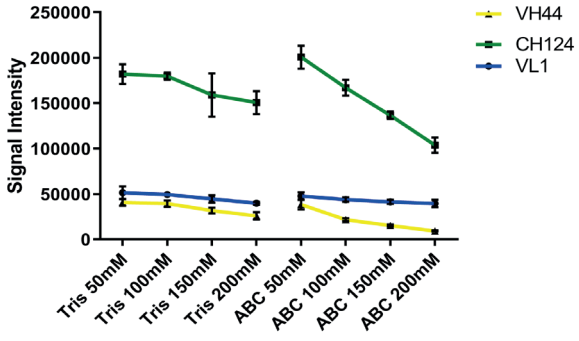


Fig. 4. Signal intensity (with standard error bars, n=3) of three peptides found in IFX versus buffer type and strength.

Figure 4 depicts the influence of buffer type and strength on the trypsin activity. Trypsin

activity can be monitored through the amount of peptides formed, which in turn is linearly correlated to signal intensity. As shown, the signal intensity diminished with increasing buffer strength. Furthermore, the rate of reduction is buffer type dependent. An increase in buffer strength in ABC resulted in a steeper decline in signal intensity of VH44 and CH124 peptides compared to Tris. Because ABC is thermally labile and because it causes a steeper decline in signal intensity for VH44 and CH124, Tris at 50 mM buffer strength was selected for use as the digestion buffer.

Comparative study

24 Samples were selected based on the concentration range (0-20 µg/mL) and the presence of ATIs. A good correlation of R^2=0.95 was retrieved between ELISA assay and LC-MS/MS (Fig. 5) using Pearson's linear regression. Furthermore, a Lin's concordance correlation coefficient [29] of ρ_c=0.83 was retrieved, indicating a substantial agreement between the two methods. Both methods reported no free IFX concentrations in the 7 patients that had developed ATIs.

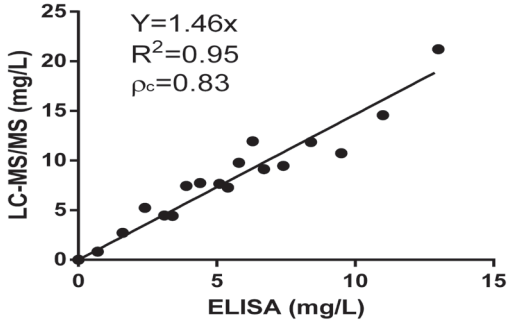


Fig. 5. Comparison of ELISA versus LC-MS/MS analysis (n=24). Seven samples containing variable levels of ATIs and no free IFX concentration.

However, LC-MS/MS IFX results were a factor 1.5× higher compared to ELISA assay, which is possibly due to the different interactions taking place during sample binding and analysis between the two assays. The ELISA assay requires two interactions, one for binding IFX to the plate and the other for IFX detection. The strength and/or efficiency of these two interactions might not be sufficient. In comparison, for the LC-MS/MS sample purification method, only one interaction is required to bind IFX to the plate. Moreover, the same between-assay

differences were observed in a recent study of five IFX ELISA assays [9]. The results showed that the Sanquin and Dynacare ELISA assays provided approximately 1.5× lower concentrations compared to Janssen, KU Leuven and LabCorp in patients treated with IFX which is similar to what was found in this study.

Validation

A linear calibration curve was established with a mean correlation coefficient of $R^2 = 0.994$ using 6 standards, 0.5 – 1 – 2.5 – 5 – 10 – 20 µg/ml analyzed over three days. The mean regression line over the three days was Y= 0.275 [±0.00538]X - 0.0006 [±0.0174] with a residual sum of squares (RSS) of 0,041 . The back calculated standard points are in concordance with EMA acceptance criteria of ±15% of the nominal value (table 2). LLOQ was greater than the acceptance criteria of 5× the noise level (Fig. 6A).

Table 2. Statistics of the back calculated concentrations of the standard curve analyzed during three days.

	Nominal concentration (µg/ml)					
	0.5	1	2.5	5	10	20
Mean	0.50	0.99	2.44	4.93	10.03	21.42
Standard deviation	0.03	0.06	0.16	0.10	0.25	1.61
Accuracy (%)[a]	-0.47	-1.00	-2.52	-1.48	0.27	7.11
Imprecision (%)[b]	6.59	5.71	6.69	2.06	2.46	7.53

[a] Accuracy: Determined as (measured conc. – nominal conc.)/nominal conc. ×100%
[b] Imprecision: Expressed as co-efficient of variation (CV)

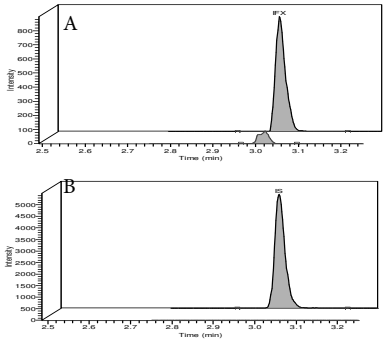


Fig. 6. SRM of (A) IFX at the back (m/z 948.53 →545.25) at LLOQ level overlaid with blank (DFHS) spiked with IS at the front. (B) IS at the back (m/z 953.53 →555.25) overlaid with blank at the front.

Selectivity and specificity were evaluated with 6 blank human serum samples and with spikes of 10µg/ml of drugs commonly found in treated patients. Because cetuximab contains near identical light chain sequence to that of IFX it was also included in the specificity test. The signal ratios obtained from the samples mentioned above, were calculated in relation to LLOQ IFX signal and IS signal respectively and are displayed in table 3.

Table 3. Selectivity tested with randomly chosen blank human serum samples, and sepecificity tested with commonly used drugs found in patients treated with IFX. Cetuximab was included do to similarities of the variable light chain with IFX.

		% Signal in relation to LLOQ signal	% Signal in relation to IS signal
Selectivity	Human serum sample 1	1.84	0.42
	Human serum sample 2	1.07	0.21
	Human serum sample 3	0.15	0.28
	Human serum sample 4	1.63	0.13
	Human serum sample 5	0.15	0.09
	Human serum sample 6	1.66	0.27
Specificity	Adalimumab	0.49	0.29
	Etanercept	5.73	0.21
	Azathioprine	3.40	0.13
	Cetuximab	1.78	0.27

As can be seen all obtained signal ratios fall well within the tolerance of 20% of LLOQ and 5% of IS signal. Carry-over peak signals were also below 20% of LLOQ for IFX and below 5% of IS signal (Fig. 6). Within-run and between-run accuracy and precision all were within acceptance criteria of <15% of the nominal concentration and 15% CV, respectively (table 4).

The % CV of IS-normalized MF calculated over the 6 blank human serum samples for QC low and QC high were 7.2% and 6.6% respectively, indicating a slight ionization enhancement but falls within the acceptance criteria of 15%. Freeze and thaw stabilities for QC low and QC high over two days were within acceptance criteria (<15% nominal concentration).

Table 4. Accuracy and precision validation data for QC's at LLOQ, Low, Medium and High levels. Within-run data were based on 5 replicates and between-run data on 3 different days.

	Precision [% CV]		Accuracy [% bias]
QC	Within- run	Overall	Overall
LLOQ	10.4	13	0.1
Low	5.5	9.4	0.5
Med	5.4	6.2	-5.7
High	5.2	6.4	-8.1

Conclusion

A target-based pre-analytical sample purification for the quantification of active form of IFX in human serum was developed. Critical parameters such as the selection of signature peptide, buffer type and strength and the capacity of streptavidin coated 96 well plate were carefully evaluated and optimized.

The method was validated according to the latest EMA guidelines and was found to be sensitive with LLOQ of 0.5 µg/mL while utilizing only 2 µL serum sample. The latter can mainly be attributed to the selective interactions of the proposed sample purification in combination with a highly sensitive mass spectrometer. Within-run and between run accuracy and precision were all within acceptance criteria. The use of stable isotopic labeled IFX bio-similar as internal standard ensured corrections for loss during sample purification, digestion and LC-MS/MS analysis. The method is easy to perform, robust and can provide a high sample throughput due to the use of the 96 well plate format. Furthermore, due to the low sample volume required for the test, lower amounts of internal standard (10 ng) and biotinylated TNF-α (0.25 µg) were needed, reducing the cost of the analysis significantly. The method showed a strong correlation with the reference ELISA and the accuracy found to be in line with previously published ELISA results.

Acknowledgment

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2.3

Simultaneous quantification of free adalimumab and infliximab in human plasma using a target based sample purification and liquid chromatography-tandem mass spectrometry.

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Therapeutic Drug Monitoring, 41 (2019) 640-647

Abstract

Therapeutic drug monitoring of tumor necrosis factor alpha (TNF-α) inhibitors such as adalimumab (ADM) and infliximab (IFX) is considered of added value for patients with systemic inflammatory diseases. In contrast to enzyme-linked immunosorbent assay (ELISA) methods, liquid chromatography tandem mass spectrometry (LC-MS/MS) methods allow simultaneous quantification of multiple target antibodies in one run and thus providing a higher sample throughput. We describe a fast sample work-up strategy for the absolute and simultaneous quantification of ADM and IFX therapeutic monoclonal antibodies in human plasma samples using a target specific sample purification in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The sample purification was based on the selective capture of ADM and IFX in human plasma or serum using biotinylated TNF-α (b-TNF-α) which was coated on a streptavidin 96-well plate. After elution, analytes were heat denatured and trypsin digested in order to obtain signature peptides for quantification. Stable isotopically labeled ADM and IFX were introduced as internal standard prior to sample purification.

The method was successfully validated following current EMA guidelines. The linear dynamic rage for both analytes were 1-32 µg/mL with an excellent mean coefficient of determination, R2 = 0.9994 for ADM and 0.9996 for IFX. Within-run and between-run imprecision and accuracy were within acceptance criteria. Cross-validation against ELISA method showed a high between method correlation R2=0.962 for ADM and R2=0.982 for IFX.

This method provides an easy, efficient and cost-effective workflow for therapeutic drug monitoring patients treated with ADM or IFX

Introduction

Adalimumab (ADM) and infliximab (IFX) are the most widely used tumor necrosis factor alpha (TNF-α) inhibitors, with more than 3.5 million people worldwide on treatment and generating an combined annual revenue above the 25 billion U.S. dollars [1-4]. These therapeutic monoclonal antibodies inactivate TNF-α by binding to its active epitope thus preventing it from attaching to its receptor resulting in the generation of inflammatory factors [5]. These antibodies have proven to be effective in achieving and maintaining remission status in patients suffering from various inflammatory diseases such as, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and inflammatory bowel disease [6-9]. Unfortunately, some patients do not experience any response, some only have a partial response while others lose their initial good response [10]. It has been established that the development of anti-drug antibodies (ADA), which enhance the clearance of these TNF-α inhibitors, results in low serum trough levels of IFX and ADM and lead to poor treatment outcome [11-14].

Therefore, there is an urgent need for efficient quantitative analytic platforms with high specificity and sensitivity for these TNF-α blocking therapeutic monoclonal antibodies. Recently, a targeted sample work-up to quantify these therapeutic antibodies has been reported using the commercially available MSIA tips which can be used with an automated liquid sample handling system or with a manual 8 channel pipet tip [15]. However, the 8 channel pipet tips principle does not allow for a high sample throughput and these tips and the liquid handlers are also relatively expensive.

Based on our previous work on the quantification of active infliximab in human serum [16], we have developed a method for absolute and simultaneous quantification of ADM and IFX concentration in plasma or serum with LC-MS/MS for therapeutic drug monitoring purposes. Furthermore, the improved method offers a higher sample throughput and ease of use due to modifications made to the elution and digestion conditions. In contrast to MSIA workflow, this method is cheaper due to the low costs of consumables and instrumentations, does not required liquid handlers and requires a much smaller sample volume (5 µL versus 35 µL) [17]. The latter also contributes to a further reduction in costs due to a reduced b-TNF-α utilization of 1 µg versus 1.5µg in the MSIA method.

Materials and methods

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) was obtained from Janssen Biologics (Leiden, The Netherlands) as lyophilized powder and was reconstituted in distilled water to a final concentration of 10 µg/µL. Stable isotopically labeled (SIL) internal standard ADM and IFX bio-similar were obtained from Promise Advanced Proteomics (Grenoble, France). Biotinylated human recombinant TNF-α was obtained from ACRO Biosystems (Newark, DE, USA). Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Scientific (Waltham, MA, USA). Streptavidin high capacity coated 96-well plate, Sigma Screen, was obtained from Sigma- Aldrich (Saint Louis, MO, USA). Protein A magnetic beads were obtained from Merck Millipore (Darmstadt, Germany). TPCK-Trypsin was supplied by Thermo Scientific as a lyophilized powder and was dissolved in acetic acid (50mM) to a concentration of 10 µg/µL, aliquoted in Eppendorf LoBind Microcentrifuge tubes and stored at -80 °C. Drug free human plasma was obtained from the local blood donor service department. All other reagents and LC-MS grade mobile phase solvents were obtained from Sigma-Aldrich.

Preparation of standards, internal standard and QC's

The combined working ADM + IFX solution at 32 µg/mL each was prepared from ADM (50 mg/mL) and IFX (10 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 and 32 µg/mL were prepared fresh from the working solution by serial dilution in drug free pooled human plasma. Internal standard SIL ADM + IFX working solution (10 µg/mL) was prepared by dilution in PBS (0.1% Tween) and stored at -80 °C. Quality Control samples (QCs) at lower limit of quantification (LLOQ) (1.0 µg/mL) , QC low (3 µg/mL), QC med (10 µg/mL) and QC high (15 µg/mL), were prepared using a ADM+IFX stock solution and pooled plasma different from that used to make the standards. Aliquots were also stored at -80 °C.

Instrumentation and chromatographic conditions

Sample purification was performed on a Vibramax 100 plate shaker, Heidolph Instruments (Schwabach, Germany). Sample denaturation and digestion was performed on Eppendorf ThermoMixer C (Hamburg, Germany). Protein A sample purification was performed on a HulaMixer™, Thermo Scientific (Waltham, MA, USA). All measurements were performed on an Ultimate 3000 UHPLC Dionex (Sunnyvale, CA, USA) coupled to a TSQ Quantiva, Thermo Scientific tandem mass spectrometer (MS) (Thermo Scientific, Waltham, MA, USA). The analytical column was an Acclaim, RSLC 120, C18, 2.1 x 100 mm, 2.2 µm particle size (Thermo Fisher). The guard column was a SecurityGuard column ULTRA C18, 2.1 mm (Phenomenex, Torrance, CA, USA). Both columns were maintained at 50 °C. The mobile phases were: (a) 0.1 % formic acid (FA) in water; (b) 0.1 % FA in acetonitrile (ACN). The UHPLC gradients in minutes per percentage of mobile phase B were 0.0 (min)/5 (% B), 3.00/35, 3.05/85, 3.95/85, 4.00/5 and 5.00/5. The flow rate was 0.6 mL/min and the run time was 5 minutes. The MS was operated in positive mode with spray voltage of 3.5 kV, Ion Transfer Tube Temperature 350 °C, vaporizer temperature 300 °C, aux gas pressure 15 Arb, sheath gas pressure 50 Arb, sweep gas pressure 0 Arb and collision gas pressure 2.5 mTorr. The precursor ions, product ions, collision energy and radio frequency (RF) lens settings are listed in Table 1 for IFX and ADM and for the internal standard.

Table 1. TSQ Quantiva SRM transitions and settings for the signature tryptic peptides of ADM, IFX and SIL internal standard (IS).

Peptide sequence	Analyte	Used as	Precursor [m/z]	Product [m/z]	Product ion	Charge	CE[a] [V]	RF[b] [V]
APYTFGQGTK	ADM	Quantifier	535.27	738.38	y7	1+	20	80
APYTFGQGT-K[13C6,15N2]	ADM	IS	539.27	746.38	y7	1+	20	80
DILLTQSPAILSVSPGER	IFX	Quantifier	948.53	545.25	y5	1+	25	110
DILLTQSPAILSVSP-GER[13C6,15N4]	IFX	IS	953.53	555.25	y5	1+	25	110
YASESMSGIPSR	IFX	Qualifier	642.80	834.41	y8	1+	25	90
YASESMSGIPS-R[13C6,15N4]	IFX	IS	647.80	844.42	y8	1+	25	90
ASQFVGSSIHWYQQR	IFX	Qualifier	598.63	754.38	y12	2+	15	80
ASQFVGSSIHWYQ-QR[13C6,15N4]	IFX	IS	601.96	759.38	y12	2+	15	80

[a] CE: Collision energy

[b] RF: Radio frequency lens

Sample preparation for LC-MS/MS analysis

Sample preparation was based on a method previously published by our group [16], with modifications to plate binding capacity, digestion conditions and ease of use. In short, b-TNF-α was coupled to a streptavidin coated 96 well plate by adding 20 µL b-TNF-α (50 µg/mL) to 180 µL PBS (0.1% Tween-20) in each well and allowed to bind for 3 hours on a plate shaker (500 rpm) at room temperature. The plate was washed three times with 200 µL PBS (0.1% Tween-20). Then, in an Eppendorf tube, 190 µL PBS (0.1% Tween-20) was added, followed by 5 µL (standard, QC or serum sample) and 5µL IS (10 µg/mL). This solution was mixed gently and transferred to the b-TNF-α pre-coated 96-well plate and allowed to bind for 3 hours at room temperature on a plate shaker (500 rpm). Then, the wells were washed three times with 200 µL PBS (0.1 % Tween-20) and eluted with 100 µL 0.1% FA 0.5% octyl glucoside by vortex mixing for 5 minutes at 1200 rpm. Extracts were transferred to a 500 µL 96-well plate, neutralized with 10µL TRIS 1M and heat denatured on a ThermoMixer C block heater set at 80 °C for 30 minutes. Samples were centrifuged at 4000g for 5 minutes and 5 µL trypsin (1 µg/µL) was added and gently mixed. Then, the plate was placed in the ThermoMixer C block heater set at 37 °C for overnight digestion. Finally, trypsin activity was stopped through the addition of 20 µL 10 % FA in ACN and 25 µL was injected and analyzed on LC-MS/MS.

Validation

The method was validated according to the latest EMA guidelines for bioanalytical method validation [18].

Comparative study

Cross-validation was performed on routine TDM plasma and serum samples of patients that were treated at our hospital (UMCU, The Netherlands) and were performed with informed consent using remnant sample material in accordance to University Medical Center Utrecht policy and ethical standards. Aliquots were sent for ELISA and RIA analysis at Sanquin (Amsterdam, The Netherlands) and analyzed according to their published methods [19-21], and the remainder was stored at -80 °C before LC-MS/MS analysis. The 25 samples for both ADM and IFX were chosen covering a range of 0-24 µg/mL and 0-31 µg/mL, respectively. Three ADA positive samples were also included in the comparison study, two from ADM and one from IFX sample set, all with values below the LLOQ.

Protein denaturation

The signal intensity of signature peptides obtained by heat denaturing method (section sample preparation for LC-MS/MS analysis) were compared against the 1,4-Dithiothreitol (DTT) method [16] and against the SMART Digest™ method from Thermo Scientific which utilizes an immobilized trypsin that allegedly offers high digestion efficiencies [22]. In short, the test was performed by pipetting 5 µL ADM + IFX (32 µg/mL in Tris 100mM, pH 8.5) in triplicates to 100 µL FA 0.1% containing 0.5% octyl glucoside and neutralizing with TRIS 1M and heat denatured at 80 °C for 30 minutes. This was compared against the same volume spiked to 100 µL 100mM Tris pH 8.5 and reduced with 5 µL DTT 100 mM at 60 °C for 30 minutes. After denaturation and reduction, samples were digested at the same time using identical trypsin concentrations. Smart Digest bulk resin was used for comparison. Here, 5 µL of the same mixed standards as above was pipetted to the buffer solution that was supplied with the kit and denaturation and digestion was performed at 70 °C for 45 minutes at 1400 RPM as indicated by the manufacturer.

B-TNF-α optimization

First, the theoretically required amount of b-TNF-α was calculated. SigmaScreen plates from Sigma-Aldrich have a theoretical binding capacity of ≥300 pmol biotin per well but are expected to have an approximately 3 times lower binding capacity for b-TNF-α due to steric hindrances thus reducing this amount to roughly 100 pmol. The combined amount of analyte plus IS at the highest concentration was 420 ng. This can be converting to molar mass: 420 ÷ 150000 × 1000 = 2.8 pmol. To round this amount up and to provide 10× molar excess, approximately 60 pmol TNF-α was needed since 1 mol IFX or ADM can bind 2 mol TNF-α. This amount (60 pmol) is within the estimated binding capacity of a SigmaScreen plate. This means that 1044 ng (60 pmol × 17400 g/mol ÷ 1000) b-TNF-α is needed to allow for 10x molar excess to bind the highest standard containing 5 µL ADM + IFX (32 µg/mL) and 5 µL IS (10 µg/mL).

Hereafter,the calculated required amount of b-TNF-α was tested with 100, 250, 500 and 1000 ng b-TNF-α in quadruplicates for increased certainty. In a high capacity 96-well plate (SigmaScreen), 2, 5, 10 and 20 µL b-TNF-α stock solution (50 µg/mL) were pipetted and filled up to 200 µL with PBS, followed by 3 hours incubation period. Five µL working solution 32 µg/mL and 5 µL SIL IS (10 µg/mL) were pipetted and purified according to the section Sample Preparation For LC-MS/MS Analysis. To determine the absolute recovery, after 3 hours of

binding, the supernatant was transferred to 50µL Protein A magnetic bead slurry to capture the unbound ADM and IFX fraction. This was left to bind overnight on a HulaMixer™ and then washed, eluted, digested and analyzed.

Sigma-Aldrich and Thermo Scientific plate comparison

Thermo Scientific high capacity streptavidin 96-well plates as alternative to the Sigma variant were also tested. A calibration curve and standards at QC low and QC high were tested following the procedures as described in the section the section Sample Preparation For LC-MS/MS Analysis using both plates and data were compared.

Results and discussion

Signature peptides

The signature peptide DILLTQSPAILSVSPGER (DILL) used for infliximab quantification was adopted from the literature [16, 23, 24]. Two additional peptides YASESMSGIPSR (YASES) and ASQFVGSSIHWYQQR (ASQ) were included as qualifiers for conformational purposes facilitated by an improved digestion conditions with increased efficiency. However, for ADM, only the signature peptide APYTFGQGTK (APY) which originates from the light chain was identified as suitable for quantification. Even though BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) results showed that this peptide is unique for ADM, total ADM analysis in drug free human serum performed in house, show that some people carry this peptide in their serum, probably as part of their immunoglobulin repertoire (data not shown). However, by using a selective purification these interfering immunoglobulins or sample matrix components were eliminated thus enabling the use of this peptide for quantification.

Optimization of protein denaturation

A new work-up using heat (80°C) to denature the protein was compared against our established work-flow which utilizes DTT at 60°C and also against a new product called SMART Digest which utilizes immobilized trypsin at 70°C.

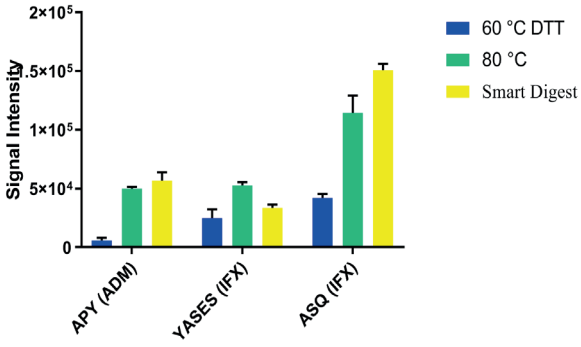

Figure 1: Method comparison using DTT, Heat (60°C) and SmartDigest (n=3, error bars represent mean with standard deviation (SD)).

Figure 1 shows that heat denaturing the proteins at 80°C, prior to digestion provides significant higher signal intensity for all examined signature peptides compared to our original method which utilized DTT at 60°C. The Smart Digest performed slightly better for APY peptide and for ASQ peptide, however, for the YASES it performed worse than heating at 80 °C. Because a complete digestion was not necessary, since a full length SIL IFX and ADM IS were used for correction and also due to the high costs of the Smart Digest kit and the relatively small signal gains that can be expected for the examined peptides, heat denaturation was chosen prior to trypsin digestion.

B-TNF-α optimization

b-TNF-α increments were tested ranging 100 ng to 1000 ng to determine the minimally required amount that delivers a high recovery and a reproducible ratio analyte/IS. Since the IS and analytes were obtained from different manufacturers, differences in tertiary structure might be present, which might result in different affinity constants resulting in variable ratios analyte/IS depending on the recovery rate. Figure 2 shows that a high recovery of 80% for ADM and 90% for IFX was obtained with 1000 ng b-TNF-α. However, the ratio analyte to IS plateaus off when a recovery above 60% was achieved, indicating that at 500 ng b-TNF-α, sufficient IS and analyte is retained to enable adequate correction for loss during sample pretreatment. Nevertheless, the highest b-TNF-α concentration was chosen to allow for maximum recovery.

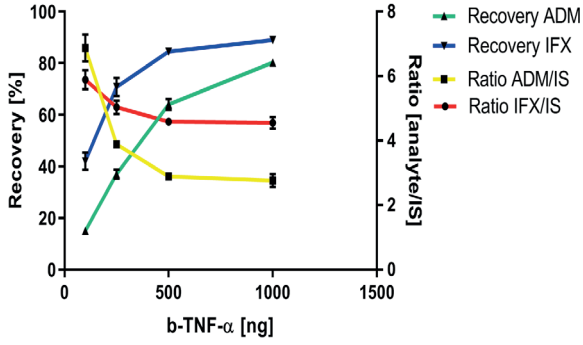

Figure 2: Binding recovery for ADM and IFX versus amount b-TNF-α used and analyte to IS ratio (n=4, error bars represent mean with SD).

Sigma Aldrich Vs Thermo Scientific plates

SigmaScreen plates with a binding capacity of ≥300 pmol, theoretically offers higher binding capacity for b-TNF-α compared to Thermo Scientific Streptavidin high capacity plates which only offer 125 pmol per well. However, the Thermo plates are cheaper than the SigmaScreen plates. Due to supply problems with the SigmaScreen plates and also since only 60 pmol b-TNF-α needed to be bound, both plates were run side-by-side and performance was compared for calibration curves, signal intensities, ratios (analyte/IS) and QC results. The calibration curves obtained using the two different plates were identical for both analytes (Fig.3).

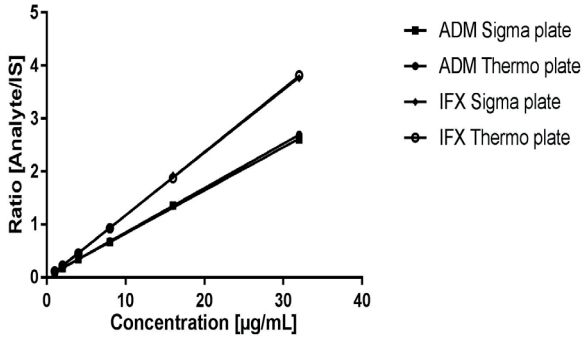

Figure 3: Calibration curves for both ADM and IFX obtained using Sigma and Thermo Scientific streptavidin coated 96-well plate.

Furthermore, the QC Low and QC High values were also within acceptance criteria (RSD and Bias <15%) for both analytes on both plates. However, when comparing the signal intensity (Fig.4), Sigma plates retained more analyte (10% for ADM and 20% for IFX) compared to the Thermo plate which would bring the estimated recovery for the Thermo plate to 70% for both ADM and IFX. Because this is higher than the 60% required for a stable analyte to IS ratio and because the QC results and the obtained calibration curves were identical between the two plates, the Thermo Scientific 96-well plates were selected for validation.

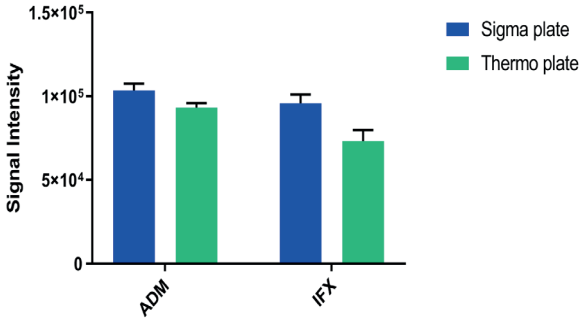

Figure 4: Relative signal (Signal Intensity IS/ average signal intensity Sigma plate × 100%) Comparison for both streptavidin coated 96-well plates (n=6, error bars represent mean with SD).

Validation

A linear calibration curve covering a range from 1 to 32 µg/mL was established with a mean coefficient of determination of R2 = 0.9994 for ADM using the signature peptide APY and 0.9996 for IFX using the signature peptide DILL. The back calculated concentrations were in concordance with EMA acceptance criteria of ±15% of the nominal value (table 2).

Table 2. Statistics of the back calculated concentrations of the standard curve for ADM (APY) and IFX (DILL) analyzed during three days.

	Nominal concentration (µg/ml)											
	1		2		4		8		16		32	
	ADM	IFX	ADM	IFX	ADM	IFX	ADM	IFX	ADM	IFX	ADM	IFX
Mean	1.11	1.12	1.75	1.79	3.87	3.89	8.54	8,22	15.75	15.67	31.98	32.31
Stdev	0.09	0.09	0.18	0.20	0.07	0.10	0.73	0,73	0.22	0.36	0.50	0.29
Accuracy	10.56	11.66	-12.52	-10.66	-3.25	-2.69	6.81	2,80	-1.53	-2.09	-0.08	0.98
Imprecision	8.09	7.97	10.57	11.07	1.81	2.54	8.57	8,89	1.42	2.30	1.56	0.90

[a] Accuracy: (measured conc. – nominal conc.) / nominal conc. × 100%

[b] Imprecision: Expressed as co-efficient of variation (CV)

The coefficient of variation at LLOQ was sufficient for both signature peptides (APY and DILL) and the signal to noise ratio was far greater than the acceptance criterion of S/N > 5 (Fig.5). The latter graph also shows the specificity of the test. Here, no baseline interference caused by endogenous peptides was observed for the pooled human serum sample at the respective retention times for both analytes and there is.

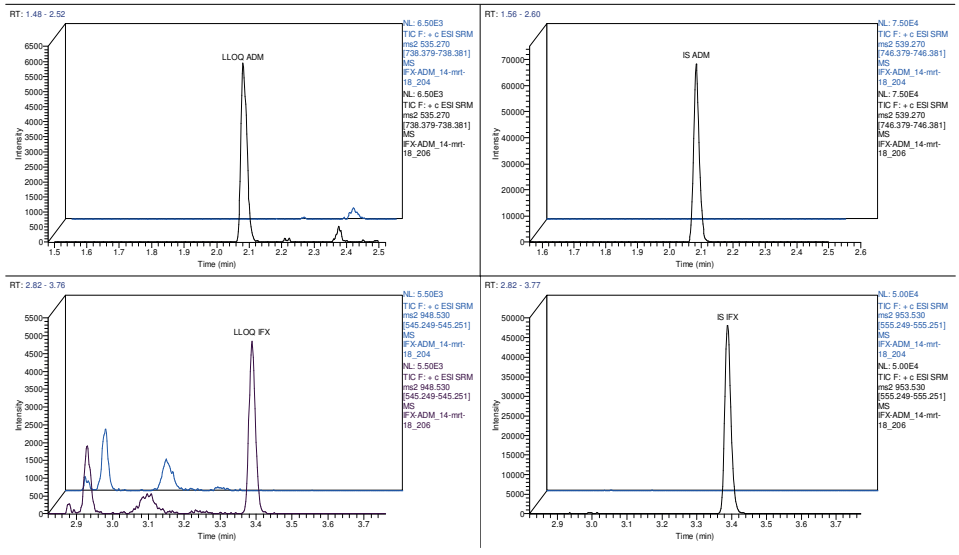

Figure 5: SRM transitions at LLOQ levels for both ADM and IFX (at the front) overlaid with blank pool serum sample (at the back); Right graphs represents IS ADM and IFX (at the front) overlaid with blank pool serum sample.

Matrix effect was evaluated by spiking plasma from six volunteers with no history of ADM or IFX treatment each at QC low (3 µg/mL) and QC high (15 µg/mL). Results are summarized in table 3 for ADM and IFX, respectively.

Table 3. ADM and IFX matrix effect tested by spiking 6 human plasma samples at QC low and QC High levels

		Measured [µg/mL]	Bias [%]	Measured [µg/mL]	Bias [%]
ADM	Sample 1	3.331	11.0	15.943	6.3
	Sample 2	3.075	2.5	16.603	10.7
	Sample 3	2.929	-2.4	16.426	9.5
	Sample 4	3.286	9.5	16.118	7.5
	Sample 5	3.191	6.4	16.095	7.3
	Sample 6	2.973	-0.9	16.079	7.2
IFX	Sample 1	3.248	8.3	15.521	3.5
	Sample 2	2.950	-1.7	16.752	11.7
	Sample 3	3.103	3.4	16.472	9.8
	Sample 4	3.457	15.2	16.626	10.8
	Sample 5	3.053	1.8	16.043	7.0
	Sample 6	2.984	-0.5	16.579	10.5

Matrix effect for both analytes were within the tolerance criteria, which states that the average determined value should be within 15% of the nominal value. Accuracy and imprecision validation for ADM and IFX were also within the acceptance criteria (Table 4).

Table 4. ADM and IFX accuracy and precision validation data for QC's at LLOQ, Low, Medium and High levels. Within-run data were based on 5 replicates and between-run data on 3 different days.

	Precision (% CV)						Accuracy (% bias)	
	Within- run		Between-run		Overall		Overall	
QC	ADM	IFX	ADM	IFX	ADM	IFX	ADM	IFX
LLOQ	8.8	6.8	4.3	0	9.8	6.8	-12.4	-7.0
Low	5.8	5.5	0	3.2	5.8	6.3	-6.0	-6.9
Med	8.8	8.4	0	0	8.8	8.4	-0.5	-2.8
High	10.3	8.5	0	0	10.3	8.5	0.2	-3.0

Auto sampler stability and freeze and thaw stability were also met EMA guidelines acceptance criteria (data not shown).

Comparative study

Sample results for ADM or IFX concentration for patients that tested positive for ADA, were as expected below the LLOQ for both ELISA and LC-MS/MS. The Pearson correlation results between methods were excellent with R2=0.962 for ADM and R2=0.982 for IFX (Fig. 6 A and C). A Bland-Altman plot showed a bias for IFX of 43.2% ± 22% (Fig. 6 D), which was in accordance with our previous findings [16].

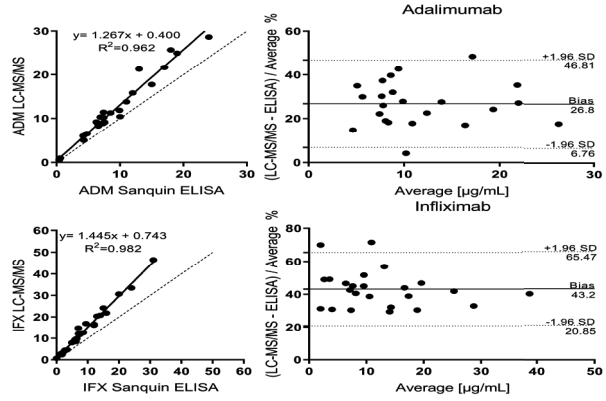

Figure 6: Pearson correlation for ADM (A) and IFX (C), ELISA vs. LC-MS/MS (n=25), Bland-Altman plot for ADM (B) and IFX (D), values lower than LLOQ were omitted.

Moreover, for ADM a bias of 26.8% ± 20% was observed which could be due to the same reasons as the IFX bias (Fig. 6 B). Namely, the sandwich type ELISA assay requires two interactions, one for binding IFX / ADM to the plate and the other for detection, whereas for the LC-MS/MS sample purification method only one interaction is required to bind the analytes to the TNF-α coated plate. As ELISA assays require two interactions, the recovery of these interaction might not be similar to the recovery obtained for the calibration curve, which contains a different matrix than the samples. Since an internal standard cannot be used for ELISA, there is no way to correct for these differences. Finally, the 95% confidence interval of the between method variations were ±20% and ±22% for ADM and IFX, respectively, which is far lower than the MSIA method which reports ±47% for ADM and ±41% for IFX (Figure 6) [15].

Discussion and conclusion

Here we present an optimized method for the simultaneous quantification of ADM and IFX in human serum or plasma. This method offers fast and easy sample preparation with high sample throughput and covers the entire therapeutic window for both components. Two different streptavidin coated 96-well plates were compared and both showed satisfactory analytical performance. The setup with 96 well plates is much cheaper than the previously described assay using MSIA® tips [15]. Importantly, there is no need for an automated liquid sample handling system to allow for high sample throughput. Furthermore, the present method utilizes only 5 µL sample versus 35 µL in the MSIA protocol [17], potentially providing the opportunity to use bloodspot samples. Moreover, higher sample volumes require more b-TNF-α, leading to a further increase in costs.

The selective purification proved to be essential in the quantification of ADM, because as we and others have found, it substantially reduces the interfering polyclonal immunoglobulin background [25]. Each step in this method was successfully optimized and validated following the latest EMA guidelines [18].

Serum samples from patients were used to cross-validate the present assay with a reference ELISA assays for ADM and IFX quantification [19, 20]. A strong correlation between the two methods was found. The bias observed for IFX was similar to our previous comparison [16], which was in line with Jansson, KU Leuven and LabCorp [26]. The ELISA assay can be more sensitive than MS methods since the enzyme horseradish peroxidase is able to convert the substrate to a colored product depending on the time allowed for the reaction. However, the fundamental problem here is that the color generated is not always directly correlated with analyte retained due to cross-reactivity. The present LC-MS/MS method allows for the measurement of the analytes within the therapeutic windows with higher degree of selectivity.

In conclusion, a rapid, cheap and selective method to quantify IFX and ADM has been developed and validated. This method can be used to monitor IFX and ADM concentrations in patients treated with these drugs.

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2.4

Quantification of T-cell binding polyclonal rabbit anti-thymocyte globulin in human plasma with liquid chromatography–tandem mass spectrometry.

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Abstract

The addition of rabbit anti-human thymocyte globulin (ATG) to the conditioning regimen prior to allogeneic haematopoietic cell transplantation (HCT) has significantly reduced the risk of graft versus host disease (GvHD) and graft failure. However, ATG has a small therapeutic window. Overexposure of ATG post-HCT hampers T-cell immune reconstitution and has been associated with increased relapse rates and viral reactivations, whereas underexposure has been associated with an increased incidence of GvHD, both of which lead to increased mortality. ATG dosing is ideally based on absolute lymphocyte count (ALC) and patients weight, however this is insufficient to prevent under- and overdosing in some patients. Therapeutic drug monitoring (TDM) of T-cell binding (active) ATG plasma levels provides a means to optimize dosing for patients at high risk for graft failure to ensure timely T-cell immune reconstitution and subsequently increase survival chances post-HCT.

This manuscript describes the first liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify the pharmacologically active fraction of polyclonal ATG in plasma. This was achieved through immunoaffinity purification of active ATG from plasma with Jurkat T-cells. After the binding and washing, samples were eluted, denatured and trypsin digested. Signature peptides originating from rabbit IgG constant heavy chain were measured with LC-MS/MS.

Critical method parameters such as binding time, ratio of Jurkat T-cells to ATG, and digestion time were optimized. Matrix effect, freeze/ thaw stability, intra and inter-assay accuracy and precision were all within EMA guidelines. The method covered the therapeutic range of ATG and was validated at an LLOQ of 1 AU/mL with an overall CV and bias of 11.8% and -2.5%, respectively.

In conclusion, we developed a LC-MS/MS-based method to quantify active polyclonal rabbit ATG in human plasma. We suggest that this novel assay can be used to monitor and optimize dosing of ATG in clinical practice.

Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative treatment strategy for both malignant and non-malignant life threatening diseases such as leukemia, lymphoma, aplastic anemia, primary immune deficiencies and inherited metabolic disorders [1]. However, graft-versus-host disease (GVHD), with mortalities as high as 50%, poses a serious side effect with incidences ranging between 20% and 70%, depending on histocompatibility mismatches, the age of recipient and the intensity of preparative regimes [2, 3]. GVHD is likely caused by the transplanted donor T-cells recognizing major and minor histocompatibility complex proteins on the recipient antigen-presenting cells [3]. Prophylaxis with polyclonal anti-thymocyte globulin (ATG) which targets different antigens expressed on e.g. T cells, B cells, natural killer cells and dendritic cells lead to depletion of these cells from the host blood and peripheral lymphoid tissues [4]. Though ATG is used as a rescue therapy for acute rejection in solid organ transplantation, its main application is in hematology to treat and prevent acute and chronic GVHD following HCT in patients with hematologic cancers [4, 5]. However, the overall survival remains similar between ATG treated and untreated patients due to increased risk of relapse and infections in ATG treated patients [6-9]. This outcome is defined by the delicate balance in timing of ATG exposures, where a high ATG exposure pre-HCT is associated with reduced GvHD and graft failures, but high exposures post-HCT are associated with increased relapse rates and reduced survival chances in patients with viral reactivations and GvHD. The presence of lytic levels of ATG post-HCT leads to poor reconstitution of donor T-cells which in turn would limit graft versus tumor effect leading to increased risk of a relapse and a reduced control of viruses and regulation of GvH-activity. European Society of Bone Marrow transplantation (EBMT) has historically recommended dosing ATG at a rate of 7.5 mg/kg over three days starting from day -3 pre-HCT in adults. Our group has shown that this dosing leads to overexposure in a majority of patients since dosing is close to graft infusion, and absolute lymphocyte counts (ALS), the most important determinant of ATG clearance at patient's body weight exceeding 40 kg, is not taken into account [6, 10]. Furthermore, to allow for improved ATG exposure an alternative dosing regimen starting at day -9 and dosing for 4 days based on ALC values and weight has been proposed. However, optimum ATG dosing of high risk patients such as those with Chronic Granulomatous Disease (CGD) or Hemophagocytic Lympho-Histiocytosis (HLH) remains difficult, possibly because of large T-cell pools in the tissues. In these patients, therapeutic drug monitoring (TDM) of free T-cell binding (active) ATG may to improve CD4+ immune reconstitution and prevention of graft failure [11].

Currently, fluorescent activated cell sorting (FACS) is used for the quantification of active

ATG in plasma [12]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is increasingly used in quantitative proteomics, because of its superior selectivity and linear dynamic range, as compared to ligand binding assays [13]. The quantification of therapeutic monoclonal antibodies (mAbs) in preclinical [14-17] and in clinical samples [18-22] has been reported using LC-MS/MS. MAbs are generally quantified by their unique signature peptide originating from either the variable region for clinical samples or sometimes from the constant region for preclinical samples [23]. However, to the best of our knowledge, the quantification of polyclonal animal based therapeutic antibodies in human plasma has not been reported previously. Here, we describe the application of LC-MS/MS to quantify therapeutic polyclonal rabbit antibodies, ATG, in human plasma samples based on constant region signature peptides.

Materials and methods

Chemicals and reagents

Rabbit ATG was obtained from Sanofi Genzyme (Cambridge, MA, USA) as a 25mg lyophilized powder which was dissolved in ultra-pure LC-MS grade water to a final concentration of 5µg/µL. Aliquots were stored at -80°C until further use. Jurkat T-cells were grown as previously described [24]. Internal standard (IS) stable isotopically labeled (SIL) peptide "LSVPTSEWQ(R $13C_6$,$15N_4$)" was obtained from Pepscan Presto BV (Lelystad, The Netherlands). Bovine serum with reference number 26010-074 was obtained from Life Technologies™ (Carlsbad, CA, USA). Human K2 EDTA plasma and serum were obtained from volunteers at the UMCU (Utrecht, The Netherlands). TPCK-Trypsin was supplied by Thermo Scientific as a lyophilized powder and was dissolved in acetic acid (50mM) to a concentration of 10 µg/µL, aliquoted in Eppendorf LoBind™ Microcentrifuge tubes and stored at -80 °C. All other chemicals, reagents and LC-MS grade mobile phase solvents were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Preparation of standards, internal standard and quality control samples

Since only a fraction of ATG in the stock standard is capable of specifically binding to T-cells, a conversion to arbitrary units (AU) was necessary. Arbitrary units are used when the real concentration is unknown. In this instance, the stock solution contains an unknown

percentage of T-cell binding ATG. Here, the ATG stock standard of 5 mg (total ATG)/mL was set as 5000 AU (active ATG)/mL as was previously described [6].

ATG working solution of 512 AU/mL was prepared by pipetting 64µL of ATG stock together with 561µl pooled plasma from five healthy donors in a LoBind™ Eppendorf tube. The highest standard solution of 32 AU/mL was prepared by combining 20µL working solution with 300µL pooled EDTA plasma.

Remaining standards 1, 2, 4, 8, 16 AU/mL were freshly prepared from the highest standard 32 AU/mL through serial dilution in pooled plasma. The internal standard SIL peptide solution "LSVPTSEWQ(R $13C_6$,$15N_4$)" at a concentration of 50 ng/mL was prepared in 0.05% Zwittergent™ 3-16 and 1% formic acid in water. Quality Control samples (QCs) at lower limit of quantification (LLOQ) (1 AU/mL), QC low (3 AU/mL), QC med (6 AU/mL) and QC high (14 AU/mL) were prepared in pooled plasma from a separate batch of healthy donors to allow for matrix variations between the standards and the controls. Aliquots were stored at -80 °C.

Instrumentation and chromatographic conditions

A HulaMixer™ from Thermo Fisher (Waltham, MA, USA) was used during immunoaffinity interaction. ThermoMixer C from Eppendorf™ (Hamburg, Germany) was used for denaturation and digestion. Centrifuge used was the Rotina 380R with a 96 well plate rotor from Hettich (Kirchlengern, Germany). All measurements were performed on a Vanquish LC coupled to a TSQ Altis mass spectrometer, Thermo Fisher (Waltham, MA, USA). The analytical column was Acquity UPLC™, BEH, C18, 2.1 x 150 mm, 1.7 µm particle size, Waters Corporation (Milford, MA, USA), the guard column was the SecurityGuard column ULTRA C18, 2.1 mm, Phenomenex (Torrance, CA, USA). Both were maintained at 50 °C. The mobile phases were: (A) 0.1% formic acid in water; (B) 0.1% formic acid in acetonitrile. The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/15 (% B), 4.5/30, 4.6/80, 5.5/80, 5.6/15 and 7/15. The flow rate was 0.45 ml/min, injection volume was 20µL and the run time was 7 min. The mass spectrometer was operated in positive mode with spray voltage of 2 kV, ion transfer tube temperature 400 °C, vaporizer temperature 350 °C, aux gas pressure 20 Arb, sheath gas pressure 50 Arb and CID Gas 2.5mTorr. The precursor ions and product ions settings are listed in table 1 for ATG and for the SIL internal standard

LC-MS/MS sample preparation

A selective sample purification method, on the basis of immunoaffinity interaction was used to capture the polyclonal active ATG fraction only. In brief, 250µL Jurkat T-cell suspension (4×10^6 cells/mL) in phosphate buffered saline (PBS buffer) and 1% human serum albumin (HSA) was pipetted in a LoBind 500µL 96 deep well plate. Subsequently, 10µL standard, QC or plasma sample was added and incubated for 2 hours at room temperature. Cells were centrifuged (500g for 5 minutes) and washed twice with 250µL PBS buffer. Each time the pellet was re-suspended and washed through vortex mixing at 1200RPM for 1 minutes and centrifugation at 500g for 5 minutes. The pellet was finally resuspended in 135µL water with a vortex at 1200RPM for 1 minutes. Hereafter, 15µL IS solution containing SIL peptide (50ng/mL in 1% formic acid, 0.05% Zwittergent™ in water) was added and mixed for a further 5 minutes, eluting bound ATG from the Jurkat T-cells. The mixture was centrifuged for 5 minutes at 500g and 100µL supernatant was pipetted into a 500µL LoBind 96 well plate. Here ATG was heat denatured at 80°C for 30 min. Then, the solution was cooled to room temperature and 10µL 1M TRIS (unbuffered) was added to neutralize the solution. Thereafter, 10µL trypsin (1µg/µL) was added and the mixture was digested at 37 °C for 1 hour. Finally, 20µL acetonitrile with 10% formic acid was added and mixed to stop the trypsin activity and dissolve the peptides. Of this solution, 20µL was injected and analyzed on the LC-MS/MS.

Signature peptide selection

ATG consists of polyclonal rabbit gamma immune globulins. Therefore, for the selection of the signature peptides used for quantification and qualification, we focused on peptides originating from the constant chains of rabbit IgG that are not endogenous to humans. Furthermore, the selected peptides should contain stable amino acids and be between 6 and 20 amino acids long. Using the amino acid sequence of rabbit IgG constant region with locus 2VUO_B (FASTA file obtained from The National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) a selection of in silico tryptic peptides was made based on predicted stability and length. Identified peptides were matched against the human genome proteins (https://blast.ncbi.nlm.nih.gov/Blast.cgi) resulting in a list of potential tryptic peptide candidates. Then, 30µL ATG stock solution (5µg/µL) in 100µL Tris buffer (100mM, pH 8.5) was denatured, digested and measured. A shallow LC-MS/MS gradient 0-40% organic in 10 minutes was used with MS operating in product ion scan mode using doubly and triply charged precursors in two consecutive runs. Two peptides with the highest signals and lowest noise were chosen for further optimization.

Effect of incubation time on signal intensity

ATG and Jurkat T-cell incubation time was optimized using 10µL ATG standard at 32 AU/mL and 250µL Jurkat T-cell suspension at 2×10^6 cells/mL. The LC-MS/MS sample preparation section described above was followed with the following modifications, LoBind Eppendorf tubes were used and an incubation time of 1, 2, 3 and 4 hours was maintained in triplicate. Upon completion of each time point, samples were centrifuged, unbound fraction was decanted and the pellet was washed. All samples were digested and measured simultaneously.

Effect of Jurkat T-cell number on signal intensity

The optimum amount of Jurkat T-cells used in relation to the highest standard 10µL of 32 AU/mL was empirically determined. Different volumes 12.5 – 25 –50 – 100 – 200 – 250µL of Jurkat T-cells suspension 10×10^6 cells/mL were pipetted in triplicates in LoBind 96 deep well plate and final volume was adjusted to 250µL with PBS. Then, 10µL of the highest standard 32 AU/ml was added to the cells and allowed to incubated for 2 hours. The samples were subsequently treated as described and signal intensity of the signature peptides and the IS was recorded.

Optimization of digestion time

In this experiment, the time required for optimum digestion of a denatured ATG standard solution (32 AU/mL) was investigated. In 18 LoBind Eppendorf tubes, 10µL ATG standard solution 32 AU/mL was mixed with 80µL water and 10µL SIL IS solution (50ng/mL, 1% formic acid, 0.05% Zwittergent™ in water). The tubes were placed on the thermomixer C at 80 °C for 1 hour. After the samples were cooled to room temperature, 10µL 1M Tris buffer was added to raise the pH to 8.5. Finally, 10µL trypsin solution (1µg/µL) was added and samples were digested in triplicates during 1, 2, 3, 4, 5 hours and overnight after which trypsin activity was stopped though the addition of 20µL 10% formic acid in acetonitrile.

Matrix effect

Matrix effect on MS-ionization and matrix-analyte interactions were investigated in this experiment. QC Low (3 AU/mL) and QC High (14 AU/mL) were prepared in PBS, human

serum (HS), human plasma (HP) and bovine serum (BS). Samples were purified with Jurkat T-cells and analyzed in five folds following the sample preparation section described above.

Validation

The method was validated following European medicines agency (EMA) guidelines for bioanalytical method validation [25].

Jurkat T-cells robustness test

Thirty EDTA plasma samples from patients treated at the UMCU with ATG have been measured with the LC-MS/MS method on two separate days. The samples were drawn in 4 mL K2 EDTA tubes, centrifuged at 4000 RPM and stored in -80 °C prior to analysis. Sample were selected based on the concentration range of active ATG. Three ranges were defined, Low (0-2 AU/mL), Med (2-7 AU/mL) and High (7-30 AU/mL) and 10 samples from each range were selected. The first run was made with a fresh batch of Jurkat T-cells. The second was from the same batch as the first but harvested a week later. The use of anonymized remnant material drawn as part of the treatment protocol and with patient's informed consent was according to University Medical Center Utrecht policy and ethical standards.

Results

Signature peptide selection

In silico digestion of rabbit IgG constant chain locus 2VUO_B produced 12 tryptic peptides with chain length between 6 and 20 amino acids long (Table 2). Four peptides contained the unstable amino acids asparagine, methionine and cysteine and were thus omitted. The remaining peptides were compared to the human Swiss-Prot database using blast. This search showed that three peptides were also endogenous to humans by having 100% match for both the query cover and identification (Table 2), and were thus dismissed. The remaining peptides were screened for signal intensity and chromatographic interference. Two peptides LSVPTSEWQR and VVSTLPIAHQDWLR were found to have the highest signal intensity and the lowest chromatographic interference and were thus chosen as signature peptides.

Table 2. Peptides with amino acids (6<n<20) obtained after in-silico digestion of rabbit constant chain locus 2VUO_B. Results for query cover and identification percentages were obtained from pBlast using human library from the Swiss-Prot database

Sequence	Mass	Stability	Query cover	Identification
EELSSR	720.35	Yes	100%	100%
AEDNYK	739.33	Yes	83%	100%
GQPLEPK	768.43	Yes	85%	100%
TARPPLR	810.49	Yes	85%	100%
DTLMISR	835.43	No	100%	100%
ALPAPIEK	838.50	Yes	100%	100%
VYTMGPPR	920.47	No	75%	83%
EQQFNSTIR	1122.55	No	100%	78%
LSVPTSEWQR	1202.62	Yes	80%	75%
VVSTLPIAHQDWLR	1634.90	Yes	92%	69%
TTPAVLDSDGSYFLYSK	1863.90	Yes	100%	88%
CPPPELLGGPSVFIFPPKPK	2120.16	No	100%	90%

Method development

ATG is a mixture of rabbit non-specific, random IgG and T-cell binding IgG. Plasma levels of the latter fraction correlate with therapeutic response [11, 26]. Therefore, a selective sample purification method was set-up to specifically quantify the T-cell binding ATG faction. Jurkat T-cells, an immortalized human T-lymphocyte line, were used to purify active ATG from plasma. After sample purification, active ATG was eluted with 0.1% formic acid containing SIL internal standard and Zwittergent™ 3-16. As internal standard a stable isotopically labeled peptide with the sequence LSVPTSEWQR[$13C_6$,$15N_4$], was used to correct for volume differences and other LC-MS/MS related variations such as injection, ionization suppression, fragmentation and signal drift. Zwittergent™, an MS compatible synthetic zwitterionic detergent, was used to reduce van der Waals interaction within and between proteins thus promoting solubility. After elution, active ATG was denatured at 80°C and trypsin digested according to our previous work [18]. The internal standard SIL peptide was measured together with two ATG tryptic peptides originating from the constant chain (Table 1).

Table 1. TSQ Altis Mass Spectrometry Conditions for SRM transitions for the signature peptide liberated from ATG after digestion with trypsin and the internal standard stable isotopic labelled peptide.

Peptide sequence	Used as	RT[a] [min]	Precursor [m/z]	Product [m/z]	Product ion	CE[b] [V]	RF[c] [V]	Dwell time [ms]
LSVPTSEWQR	Quantifier	2.75	601.81	903.43	Y7	20	70	250
LSVPTSEW-QR[13C6,15N4]	IS	2.75	606.81	913.43	Y7	20	70	50
VVSTLPIAHQDWLR	Qualifier	4.80	817.95	1135.60	Y9	30	100	300

[a]RT: Retention time
[b]CE: Collision energy
[c]RF: Radio frequency lens

Effect of incubation time on signal intensity

To assess the incubation time required for optimum immunoaffinity, 10µL of the highest ATG standard of 32 AU/mL was incubated with 250µL Jurkat T-cell suspension 2 $\times 10^6$ cells/mL for up to 4 hours.

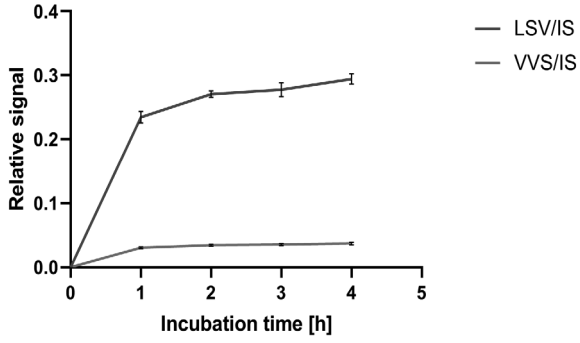

Figure 1: Effect of incubation time (x-axes in hours) on relative signal intensity (y-axes) ratio signature peptide LSV and VVS divided by internal standard (IS). Results represent mean and SD of n=3

Figure 1 shows that at 2 hours incubation a stable plateau was reached. Only a slight signal increase <10% was observed between the 2 and 4 hour time points. Therefore, a 2 hour incubation period was used in further experiments. As the signal intensity of LSV was approximately six times higher compared to VVS signature peptide, LSV was chosen as the quantifier and VVS as qualifier peptide.

Effect of Jurkat T-cell number on signal intensity

The optimal number of Jurkat T-cells needed to capture active ATG from 10µL of the highest standard 32 AU/mL was established. As can be seen from figure 2, both signature peptides (LSV and VVS) reached a plateau when 1 million cells were used. Furthermore, the coefficient of variation was smaller when more cells were used. After centrifugation, the large compact pellets were more firmly attached to the 96 well plate surface, thus minimal sample loss incurred during the washing steps.

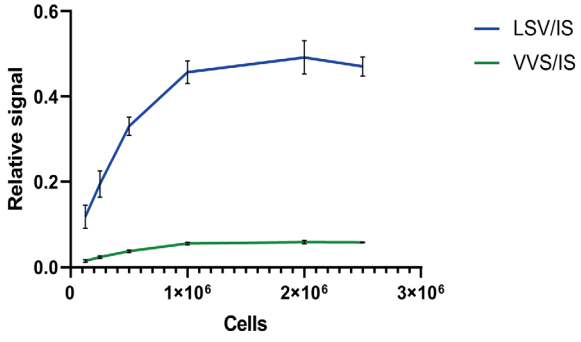

Figure 2: Effect of the number of cells in 96-well plate (x-axes) on the relative signal intensity of the highest ATG standard 32 AU/mL (y-axes). Incubation time with Jurkat T-cells was 2 hours, Results are means and SD of n=3

Optimization of digestion time

Optimum digestion time was investigated with ATG spiked in the same TRIS buffer solution as described in LC-MS/MS sample preparation. Triplicates were digested during 1, 2, 3, 4, 5 hours and overnight. Data obtained shows that 1 hour digestion provides similar signal intensity as overnight digestion (Fig. 3).

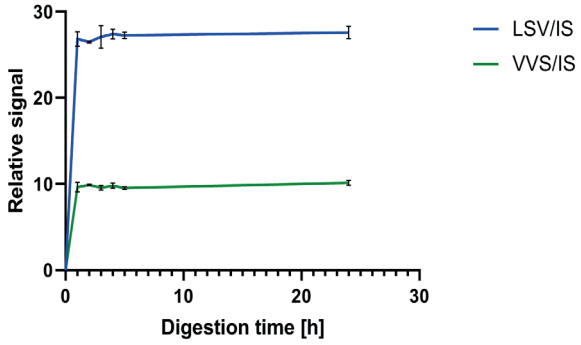

Figure 3: Effect of digestion time (x-axes in hour) on the relative signal intensity of the highest ATG standard 32 AU/mL. Results are means and SD of n=3

Matrix effect

Cross reactivity of ATG with matrix components such as endogenous immunoglobulins, coagulation factors or albumin could lead to low T-cell binding ATG recoveries. Furthermore, matrix could also interfere with MS-ionization leading to signal suppression and thus result in loss of sensitivity. To correct for this, an additional sample clean-up would be required to eliminate the interfering matrix component.

As can be seen from spiking experiments shown in figure 4, there are no significant differences between the various matrices tested in both the low and high concentration range. Therefore, analysis can be performed in both serum or EDTA plasma. Furthermore, differences in ionization were tested by comparing the internal standard (IS) signal originating from ATG spiked in PBS to the IS signal obtained from the various sample matrices (figure 5). No ionization suppression or enhancement was observed between the various matrices tested and PBS, for both low and high ATG spiked concentrations.

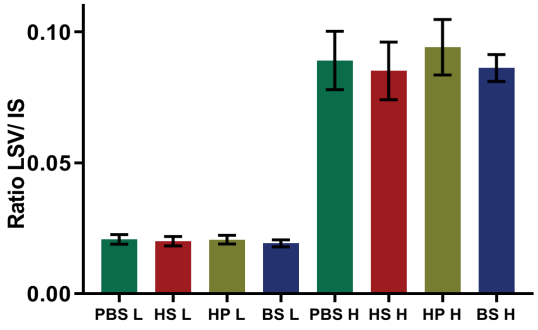

Figure 4: Comparison of relative signal intensity (y-axes) obtained from various matrixes (PBS = phosphate buffered saline, HS = human serum, HP = human plasma, BS = bovine serum) spiked with ATG at low (3 AU/mL) and high (14 AU/mL) concentration (x-axes). Results are means and SD of n=5

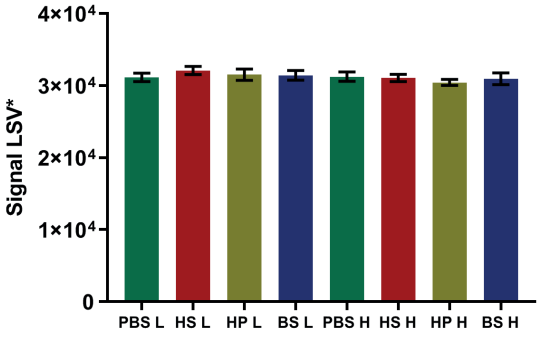

Figure 5: Comparison of internal standard signal intensity (y-axes) obtained from various matrixes. Results are means and SD of n=5

Validation

The LLOQ for the quantifier peptide LSV was validated by comparing the signal at LLOQ level (1 AU/mL) to the noise obtained from a negative human plasma sample (Fig. 6 A, B). Here, a signal to noise ratio of around 500 was obtained, which was significantly above EMA guidelines of S/N >5. This could potentially allow for an even lower detection limit if needed for future studies. Furthermore, blank human plasma did not contain any detectable peak

at the retention time of the internal standard thus ensuring a high selectivity (Fig. 6 C, D). Calibration curve was linear between 1 and 32 AU/mL with $R^2 > 0.999$. Carry over was 0.02% of LLOQ signal and overnight auto sampler stability was evaluated by reinjecting LLOQ, QC Low and QC High after 5 days, the difference between results were <10% for all values which is within guidelines. Freeze/ thaw stability of ATG was validated during three freeze/thaw cycles in fivefold using QC Low and QC High. Both QC Low and QC High were stable during the three cycles, bias and CV did not exceed the threshold of 15%. Finally, accuracy and precision were validated during three days with LLOQ, QC Low, QC Medium and QC High measured in fivefold (Table 3). Both parameters were in concordance with guidelines. An additional matrix effect experiment was carried out by spiking plasma from 6 volunteers at QC low and QC high concentration. Back calculated concentrations were all within 15% bias of the true value (Table 4).

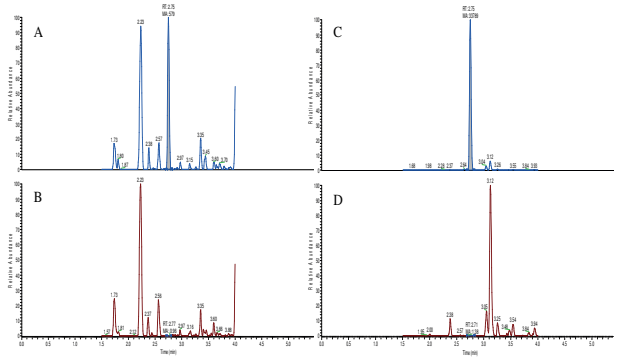

Figure 6: Left side, LC-MS/MS chromatogram of ATG standard 1 AU/mL (A) and chromatogram of negative human plasma containing IS (B) both measuring LSVPTSEWQR peptide with SRM transition 601.81 → 903.43. Right side, chromatogram of internal standard (C) and chromatogram of blank (D) measuring the stable isotopically labeled peptide LSVPTSEWQR[13C6,15N4] with SRM transition 606.81 → 913.43

Table 3. Accuracy and precision validation data for ATG QC's at LLOQ, Low, Medium and High levels. Within-run data were based on 5 replicates and between-run data on 3 different days. Data based on the quantifier peptide LSV measuring 601.81→903.43 transition.

	Precision (% CV)			Accuracy (% bias)
QC	Within- run	Between-run	Overall	Overall
LLOQ	10.7	5.0	11.8	-2.5
Low	7.6	7.5	10.7	0.9
Med	7.7	2.5	8.1	-3.1
High	5.2	9.0	10.4	-2.9

Table 4. Matrix effect test, 6 human plasma samples spiked with ATG at QC Low (3 AU/mL) and QC High level (14 AU/mL).

Sample nr.	Measured [mg/L]	Bias [%]	Measured [mg/L]	Bias [%]
1	2.99	-0.3	13.64	-2.6
2	2.93	-2.3	13.03	-6.9
3	2.86	-4.7	14.38	2.7
4	3.25	8.3	13.91	-0.6
5	2.80	-6.7	12.87	-8.1
6	2.56	-14.8	12.60	-10.0

Jurkat T-cells robustness test

Plasma samples from 30 patients treated with ATG have been analyzed with a freshly made Jurkat T-cell batch and the same batch harvested 1 week later for the second day run. Pearson's regression (Fig. 7A) shows that reproducible results were obtained (r^2= 0.995). Bland-Altman plot (Fig. 7B) indicates an overall bias of -9% which is within acceptance criteria of 15%. At the lower concentration scale we see a larger variability but this is to be expected since values at the LLOQ range (1 AU/mL) are allowed a bias ≤20%. Results obtained from the quantifier ion measurement were compared to those from the qualifier ion and a good correlation was found. The results from the lower end 0- 3 AU/mL were not as strong as the higher end due to the higher detection limit of the qualifier peptide This data shows that patients treated with ATG can successfully be monitored in the relevant concentration range with LC-MS/MS.

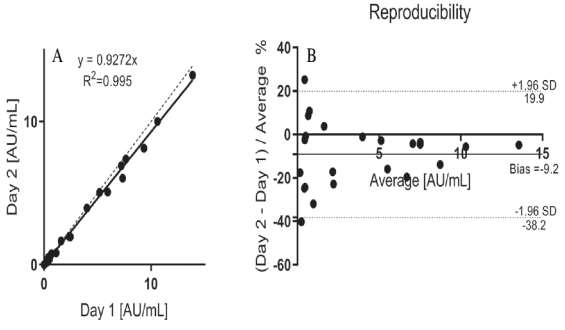

Figure 7: Pearson's regression (A) and Bland-Altman plot (B) of reproducibility data obtained during the analysis of active ATG in patients EDTA plasma samples on two separate days n=30.

Conclusion and discussion

Here we describe the first LC-MS/MS method to quantify active ATG. LC-MS/MS allows for increased selectivity by directly measuring the signal intensity of LSVPTSEWQR and VVSTLPIAHQDWLR peptides, which originate from the constant chain and are exclusively present in rabbit IgG. The qualifier peptide VVS provided a means to check the results obtained from the quantifier peptide LSV as to ascertain that no isobaric interferences were present. This method allows samples to be quantified for research purposes and routine therapeutic drug monitoring. Furthermore, due to its linear dynamic range 1 - 32AU/mL, it allows quantification to be performed without the need of multiple sample dilutions as in the case with FACS methods. The assay does not depend on fluorescent tagged antibodies for detection and is easy to perform requiring only two washing steps after incubation. The use of 96-well plate format enables high throughput analysis and the small sample volume required for analysis 10µL is less invasive for the younger patients.

The limitations of the LC-MS/MS method is the introduction of the stable isotopically labeled internal standard in conjunction with the elution step following the immunoaffinity capture. This means that the internal standard cannot correct for loss during the binding and washing steps. However, since the pallet remains tightly bound to the plate after centrifugation, the within-run error was found to be <15% CV. Furthermore, by performing duplicate analysis of the sample it is possible to monitor the efficiency of this step.

The therapeutic window for ATG has been described in pediatric patients receiving bone marrow and cord blood after myeloablative conditioning and adult patients receiving peripheral blood stem cells after reduced intensity conditioning. Still, the therapeutic window remains to be determined in more settings, including T-cell depleted and haplo-transplants.

Furthermore, the proposed population pharmacokinetics models for children and adults adequately predict concentration-time curves [6]. There is however some unexplained variability in clearance, more profound in adults compared to children. This variability can be eliminated by performing TDM.

TDM can also be used in patients at high risk for graft failure and GVHD, where more extreme exposures to ATG may be needed [11]. By monitoring and optimizing the concentration of active ATG, immune reconstitution of high risk patients can be improved and graft failure can be prevented both of which have been associated with increased overall survival.

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2.5

Quantification of total dinutuximab concentrations in neuroblastoma patients with liquid chromatography tandem mass spectrometry.

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Abstract

Neuroblastoma is one of the most commonly found solid tumors in children. The monoclonal antibody dinutuximab (DNX) targets the sialic acid-containing glycosphingolipid GD2 expressed on almost all neuroblastoma tumor cells and induces cell lysis. However, the expression of GD2 is not limited to tumor cells only, but is also present on central nerve tissue and peripheral nerve cells explaining dinutuximab toxicity. The most common adverse reactions are pain and discomfort, which may lead to discontinuation of the treatment. Furthermore, there is little to no data available on exposure and effect relationships of dinutuximab. Therefore, we developed an easy method in order to quantify dinutuximab levels in human plasma.

Ammonium sulfate (AS) was used to precipitate all immunoglobulins (IgG's) in human plasma. After centrifugation, supernatant containing albumin was decanted and the precipitated IgG fraction was re-dissolved in a buffer containing 0.5% sodium dodecyl sulfate (SDS). Samples were then reduced, alkylated and digested with trypsin. Finally, a signature peptide in complementarity-determining region 1 of DNX heavy chain was quantified on LC-MS/MS using a stable isotopically labeled peptide as internal standard. AS purification efficiently removed 97.5% of the albumin fraction in the supernatant layer.

The validation performed on DNX showed that within-run and between- run coefficients of variation (CV's) for lower limit of quantification (LLOQ) were 5.5% and 1.4%, respectively. The overall CV's for quality control (QC) Low, QC Med and QC High levels were <5%. Linearity in the range 1 – 32 mg/L was excellent (r^2>0.999). Selectivity, stability and matrix effect were in concordance with EMA guidelines.

In conclusion, a method to quantify DNX in human plasma was successfully developed. In addition, the high and robust process efficiency enabled the utilization of a stable isotopically labelled (SIL) peptide instead of SIL DNX, which was commercially unavailable.

Introduction

Neuroblastoma (NB) is the third most common childhood cancer with a prevalence of 10.2 cases per million children under the age of 15 years [1]. NB is an embryonic cancer of the post-ganglionic sympathetic nervous system which is usually formed in nerve tissues of the adrenal gland, neck, chest or spinal cord [2]. Early diagnosis of high risk NB is very difficult, however depending on the stage of the disease, tumors can clearly be seen as a lump under the skin. Treatment strategies of NB is dependent on the risk group it has been categorized to, since some cases of NB can show spontaneous and complete regression [1, 3-5]. However, the long-term survival of high risk NB is between 35 to 45% despite multimodal treatment [6-8]. Therefore, a new treatment strategy based on the chimeric, mouse-human, monoclonal antibody dinutuximab (CH14.18/SP2/0; Unituxin; DNX) has been developed to target and eradicate remaining NB cells in order to prevent relapse [9, 10]. DNX was approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) in 2015 and is used in combination with granulocyte-macrophage colony-stimulating factor, interleukin-2, and isotretinoin [10, 11]. This therapeutic antibody targets the sialic acid-containing glycosphingolipid GD2 which is overexpressed on almost all NB tumor cells and induces cell lysis trough complement-dependent cytotoxicity and cell necrosis and apoptosis trough antibody-dependent cell-mediated cytotoxicity [8, 10, 12, 13].

However, treatment with DNX causes neuropathic pain due to GD2 presence on nerve cells, this necessitates the use of opioids prior to, during and for 2 h after administration of DNX in order to manage pain [10]. A method to quantify DNX levels in plasma can potentially lead to new insights for personalize dosing to increase efficacy and reduce toxicity. Furthermore, it has been estimated that up to 37% of patients develop anti-drug antibodies (ADA) which could have a profound impact on drug clearance [14-16], and thus therapeutic drug monitoring of these patients is of great value.

To date, three ligand binding assays are described, two based on anti-idiotypic antibodies to DNX and the other is a cell-based enzyme-linked immunosorbent assay (ELISA) using GD2 expressing melanoma cell line [17-19]. However, the generation of these cell lines and antibodies requires specific skills and facilities.

Recently, the introduction of highly-sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) technology has enabled monoclonal antibody quantification. LC-MS/MS possesses notable advantages over ELISA methods such as faster assay setup times, wider linear dynamic rage and most importantly higher selectivity [20, 21]. Therefore, we have developed an easy method to quantify total DNX in plasma using a novel sample preparation in combination with tandem mass-spectrometry analysis.

Materials and methods

Chemicals and reagents

Dinutuximab (CH14.18/SP2/0; Unituxin™; DNX) was obtained from United Therapeutics Europe, Ltd (Chertsey, United Kingdom) as a solution of 3.5 mg/ml. Dinutuximab beta (CH14.18/CHO; Isquette™; DNX-β) was obtained from Retschler Biotechnologie GmbH (Laupheim, Germany) as a solution of 4.5 mg/ml. As internal standard (IS) stable isotopically labeled (SIL) peptide "ASGSSFTGYNMNWV(R $13C_6$,$15N_4$)" was obtained from Pepscan Presto BV (Lelystad, The Netherlands). TPCK-Trypsin was supplied by Thermo Scientific (Waltham, MA, USA) as a lyophilized powder and was dissolved in acetic acid (50mM) to a concentration of 10 µg/µL, aliquoted in Eppendorf LoBind™ Microcentrifuge tubes and stored at -80 °C. All other chemicals, reagents and LC-MS grade mobile phase solvents were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Preparation of standards, internal standard and quality control samples

The working DNX standard solution (32 µg/mL) was prepared by pipetting 30 µL stock solution Unituxin (3.5 µg/µL) and 180 µl pooled plasma in a LoBind™ Eppendorf tube to obtain a concentration of 500 µg/mL. This solution was further diluted to 32 µg/mL in pooled plasma and aliquots were stored at -80 °C. Standard concentrations of 1, 2, 4, 8, 16 and 32 µg/mL were freshly prepared from the working standard solution by serial dilution in pooled plasma. The working internal standard SIL peptide solution "ASGSSFTGYNMNWV(R $13C_6$,$15N_4$)" at a concentration of 0.5 mg/L was prepared in tris(hydroxymethyl)aminomethane (Tris) buffer pH 8.5, 100mM containing 0.5% octyl glucoside (OG). Quality Control samples (QCs) at lower limit of quantification (LLOQ) (1 µg/mL), QC low (3 µg/mL), QC med (15 µg/mL) and QC high (25 µg/mL) were prepared in pooled plasma from a separate batch. Aliquots were stored at -80 °C.

Instrumentation and chromatographic conditions

Sample reduction, alkylation and digestion was performed on Eppendorf ThermoMixer C. All measurements were performed on an Ultimate 3000 UHPLC Dionex (Sunnyvale, CA, USA) coupled to a TSQ Quantiva, Thermo Fisher (Waltham, MA, USA). The analytical column was

Acclaim™, RSLC 120, C18, 2.1 x 100 mm, 2.2 µm particle size, Thermo Fisher (Waltham, MA, USA), The Guard column was the SecurityGuard column ULTRA C18, 2.1 mm, Phenomenex, (Torrance, CA, USA). and were maintained at 50 °C. The mobile phases were: (A) 0.1% formic acid in water; (B) 0.1% formic acid in acetonitrile. The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/2 (% B), 7.5/24, 7.6/85, 8.5/85, 8.6/2 and 10.5/2. The flow rate was 0.6 ml/min and the run time was 10.5 min. The mass spectrometer was operated in positive mode with spray voltage of 3.5 kV, ion transfer tube temperature 350 °C, vaporizer temperature 300 °C, aux gas pressure 15 Arb, sheath gas pressure 50 Arb, collision energy 30 V, collision gas pressure 2.5 mTorr and radio frequency (RF) lens 110 V. The precursor ions and product ions settings are listed in table 1 for DNX and for the SIL internal standard.

Table 1. Mass Spectrometer conditions for selected reaction monitoring (SRM) transitions for the signature peptide (liberated from DNX after digestion with trypsin) and the SIL internal standard

Peptide sequence	Used as	Precursor [m/z]	Product [m/z]	Product ion
ASGSSFTGYNMNWVR	Qualifier	838.88	819.39	Y6
ASGSSFTGYNMNWVR	Quantifier	838.88	1039.47	Y8
ASGSSFTGYNM(O)NWVR	Oxidation check	846.88	1055.47	Y8
ASGSSFTGYNMNWVR	Qualifier	838.88	1140.52	Y9
ASGSSFTGYNMNWVR [13C6,15N4]	Qualifier	843.90	829.39	Y6
ASGSSFTGYNMNWVR [13C6,15N4]	Quantifier	843.90	1049.47	Y8
ASGSSFTGYNM(O)NWVR [13C6,15N4]	Oxidation check	851.90	1065.47	Y8
ASGSSFTGYNMNWVR [13C6,15N4]	Qualifier	843.90	1150.52	Y9

Sample preparation for LC-MS/MS analysis

Ammonium sulfate (AS) protein precipitation method was chosen because of its inherent simplicity, high sample throughput and fast work flow (Fig. 1). In brief, 10 µL (sample, standard or QC) was diluted with 90 µL Tris (50 mM, pH 8, 0.5% OG) in 1 mL LoBind 96 well plate. Then, 70 µL AS (saturated) was added to each sample followed by 1 minute mixing at room temperature. The 96 well plate was centrifuged at 4000 G for 5 minutes to collect the IgG pellet at the bottom. The supernatant containing albumin was decanted and the pellet was re-dissolved in 50 µL Tris (100 mM, pH 9, 0.5% sodium dodecyl sulfate (SDS), 20 mM 1,4-dithiothreitol (DTT)). Then, the well plate was placed in a ThermoMixer at 60 °C, 1000 RPM for 30 minutes to reduce the disulfide bonds. Samples were alkylated by adding 20 µL iodoacetamide (IAA) (100mM dissolved in ultrapure water) and placed on a ThermoMixer at 37 °C for 30 min in the dark. Then, 150 µL ultrapure water was added and mixed for 1 minute

to dilute the SDS and IAA. After mixing, methanol was added to precipitate the IgG fragments and the well plate was centrifuged at 4000 G for 5 minutes. The supernatant containing SDS and IAA was decanted. Then, 90 µL IS working solution was added, followed by 10 µL Trypsin (2 µg/µL) and the samples were placed on the ThermoMixer for overnight digestion at 37 °C, 800 RPM. Trypsin activity was stopped by adding 30 µL 10% formic acid, 5% trifluoroacetic acid (TFA) in acetonitrile. Finally, 25 µL was injected on an LC-MS/MS.

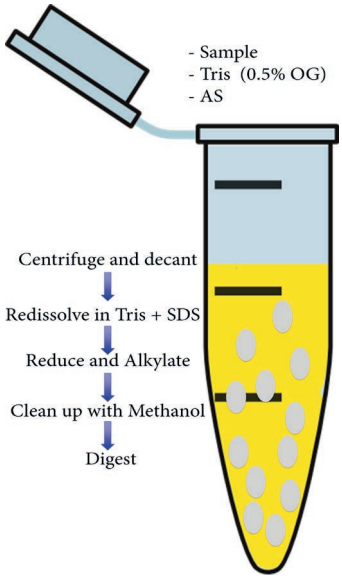

Figure 1: Sample purification workflow using AS

Signature peptide selection

Tryptic digestion is regularly used to obtain peptides to enable low quantification levels of therapeutic monoclonal antibodies (mAb) in plasma with a triple quadrupole mass spectrometer. DNX sequence was obtained from the international immunogenetics information system® (http://imgt.org). After in silico digesting the variable chains with the online tool from institute of systems biology (http://db.systemsbiology.net), potential signature peptide candidates having amino acids in the range of 6<n<20 were identified. These amino acids were then screened using pBlast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Finally, the retention time (RT) of the surrogate peptide candidates were identified on the Q Exactive™ (Thermo Scientific) and the signal intensity of all peptides were compared.

Albumin determination

After sample purification with AS, the remaining concentration albumin in the pellet was measure by means of immunonephelometry on the BN ProSpec™ System (SIEMENS). In brief, 200 µL plasma from a healthy donor was diluted with 1800 µL Tris (50 mM, pH 8, 0.5% OG) in a test tube in triplicates. Then, 1400 µL AS (saturated) was added to each tube followed by 1 minute mixing at room temperature. Then the test tubes were centrifuged at 4000 G for 5 minutes to collect the IgG pellet at the bottom. The supernatant was decanted to the waste and the pellet was re-dissolved in 200 µL phosphate-buffered saline (PBS). The remaining albumin in the pellet and in the original plasma sample were measured on the BN ProSpec™.

AS concentration and DNX recovery

10 µL standard 6 (32 mg/L) was pipetted 12 times in LoBind 96 deep well plate followed by 90 µL Tris (pH 8, 0.5% OG). Then, 50, 60, 70 and 80 µL saturated AS was added in triplicates followed by mixing and centrifugation. Samples were then prepared and analyzed.

Methanol concentration and DNX recovery

10 µL standard 6 (32 mg/L) was pipetted 18 times in LoBind 96 deep well plate followed by 90 µL Tris (pH 8, 0.5% OG). Samples were then prepared according to sample preparation section with the following modifications; following alkylation 0, 50, 100, 150, 200, 250 µL water was added in triplicates followed by 550, 500, 450, 400, 350, 300 µL methanol also in triplicates.

Comparison between DNX and DNX-β

A test was performed to determine whether concentrations of DNX and DNX-β were similar. A DNX-β control sample was prepared at a concentration of 15 mg/L and was run in duplicate against DNX calibration curve. DNX standards and the DNX-β control sample were prepared as described in sample preparation section.

Patient infusion samples

After obtaining parental informed consent, eight peripheral blood samples of 2 mL (in EDTA-treated tubed) were drawn from one pediatric patient each time before and after DNX infusion. The patient was given a daily infusion of 14.01 mg DNX for 10 – 20 hours during a 4 day treatment period. Peripheral blood samples were centrifuged at 600 G for 15 minutes. Following centrifugation, the supernatant (plasma) was carefully removed from the cell pellet. Plasma was aliquoted into 1-2 volumes of 0.5mL and immediately stored at -80°C. After storage, samples were analyzed in duplicates according to the procedure described in chapter 2.4.

Validation

The method was validated according to EMA guidelines which dictate the investigation of parameters such as, LLOQ, linearity, selectivity, matrix effect, carry-over, auto sampler stability, freeze/thaw stability, within-run and between-run precision and accuracy [22]. LLOQ was chosen based on the reference ELISA used to generate pharmacokinetic data for FDA approval [23] and was determined by comparing the signal obtained from standard 1 (1 mg/L) against a pooled normal human plasma after sample preparation according to the above described method. The acceptance criterion is that the signal of standard 1 (LLOQ) should be at least 5 times higher than the signal obtained for the pooled normal human plasma at the RT of the signature peptide. The linear dynamic range of the standard curve was established based on theoretical peak levels that would be obtained in patients and was investigated by measuring 6 standards at concentrations 1.00, 2.00, 4.00, 8.00, 16.00, 32.00 µg/mL for 3 days. The acceptance criterion for the back calculated concentration for LLOQ was 20% of the nominal value and for the remaining standard 15% of the nominal value. Selectivity was tested by evaluating 10 human plasma samples from healthy donors and comparing the MS signal intensity at the RT of the signature peptide against LLOQ signal. The noise signal intensity obtained for the blank plasma samples at the RT of DNX signature peptide should be less than 20% of the LLOQ signal. Matrix effect was tested by spiking human plasma samples from healthy donors at QC low (3 mg/L) and QC high (25 mg/L) and determining the concentration with the calibration curve. The back calculated concentration should be within 15% of the nominal value. Carry-over was tested by injecting digested pooled normal human plasma sample after a high standard. The acceptance criterion, in this case, was a signal obtained at the RT of DNX signature peptide of less than 20% of the LLOQ and a signal obtained for the IS under 5%. Auto sampler stability was tested by re-injecting the validation

samples the next day and comparing the results to those of the day before. Freeze and thaw stability was validated by analyzing a QC Low and QC high sample in 5-fold during 3 days at which samples were thawed and tested, and the remaining samples were stored again at -80 °C, and subjected to the same procedure next day. Within-run and between-run precision and accuracy was evaluated during 3 days by analyzing LLOQ (1 µg/mL), QC Low (3 µg/mL), QC Med (15 µg/mL) and QC High (25 µg/mL) in 5 folds each day. The data obtained for each concentration was evaluated with single factor ANOVA. Accuracy was expressed as percentage bias. Within-run and between-run precision was expressed as percent coefficient of variation (% CV) and was calculated by taking the squared root of the mean squares (MS) and dividing this by the overall mean concentration times 100%.

Results and discussion

Method development

A novel sample preparation method was developed based an optimized combination of established methods from literature [20, 24-27]. AS purification was found to be a fast, easy and efficient way to remove plasma albumin which comprises of approximately 60% of total plasma proteins. Furthermore, the protein pellet could easily be re-dissolved in working buffers, suggesting that AS did not denature the IgG's and kept the tertiary structure intact. The use of a MS compatible non-ionic surfactant, octyl glucoside (OG), with AS aided in the removal of phospholipids which are notorious MS-ionization suppressants. SDS is a widely used inexpensive ionic detergent, and is very efficient in protein unfolding and solubilization when used under reducing conditions in presence of DTT. However, SDS is not compatible with trypsin nor with MS thus removal prior to these steps is essential. Using our method, SDS was efficiently removed by protein precipitation with methanol, as SDS remains in solution in the aqueous layer. This was verified by evaporating the supernatant layer under nitrogen. Upon the addition of water, foam was clearly visible after agitating the test tube. Methanol precipitation also allowed for efficient removal of the remaining salts. The internal standard was a SIL peptide ASGSSFTGYNMNWV(R $13C_6$,$15N_4$) and was introduced to the samples prior to digestion. The internal standard allowed for correction of ionization suppression and injection volume differences during MS analysis. Importantly, digestion with trypsin needed to be reproducible between different patients because the SIL peptide cannot correct for digestion efficiency. Here, we found that the protein pellet was completely dissolved in all patients containing different levels of IgG's during matrix effect studies

Signature peptide selection

In silico digestion of the variable light (VL) and heavy chain (VH) provided 10 potential candidates that were between 6 to 20 amino acids long and that could possibly be used as signature peptide (table 2).

Table 2. Peptides with amino acids (6<n<20) obtained after in-silico digestion of the variable chains. Results for query cover and identification percentages were obtained from pBlast using human library from Swiss-Prot database.

Location	Sequence	Mass	Query cover	Identification
VH24	ASGSSFTGYNMNWVR	1676.7485	100%	73%
VH44	SLEWIGAIDPYYGGTSYNQK	2262.0713	80%	63%
VH68	ATLTVDK	747.4247	100%	86%
VH75	SSSTAYMHLK	1124.5405	100%	88%
VL1	EIVMTQSPATLSVSPGER	1901.9637	100%	100%
VL19	ATLSCR	650.3290	100%	100%
VL25	SSQSLVHR	913.4850	75%	100%
VL33	NGNTYLHWYLQKPGQSPK	2131.0719	94%	82%
VL60	FSGVPDR	777.3889	85%	100%
VL67	FSGSGSGTDFTLK	1303.6164	100%	100%

After preforming a protein blast search three peptide candidates (VL1, VL19 and VL67) were found to be endogenous to humans and were dismissed. VL 33 was also dismissed because it contained asparagine followed by glycine. Glycine, a small amino acid group is not capable of shielding asparagine from deamination reaction. The remaining candidates were screened using high resolution mass spectrometry after tryptic digestion (data not shown). Signals for peptide VH68 and VL60 were found to be low, probably due to charge interference caused by the aspartic acid group (D) near the trypsin digestion sites (K and R). Peptide VH24 (ASGSSFTGYNMNWVR) and VH44 (SLEWIGAIDPYYGGTSYNQK) from the complementarity-determining region (CDR) 1 and CDR 2, respectively were found to have the highest signal intensity and both were optimized for collision energy and RF lens settings on the triple quadrupole. After optimizing the digestion condition, the signal intensity of VH44 was found to be too low to allow for quantification at the required LLOQ of 1 µg/mL. Therefore, VH44 peptide was omitted and the validation was performed on the VH24 peptide. Although VH24 peptide contains a methionine group, no oxidation peaks were found after overnight digestion (data not shown).

Level of albumin remaining after AS purification

The albumin concentration of the untreated plasma sample and the AS pellet re-dissolved in PBS buffer were measured in triplicate by means of immunonephelometry. The untreated plasma sample had a mean concentration albumin of 39,5 g/L with a standard deviation (SD) of 0.72 g/L and the AS pretreated plasma sample had a mean remaining albumin concentration of 0.98 g/L with an SD of 0.1g/L. This translates to a highly efficient depletion of 97.5% albumin with AS pretreatment (Fig. 2).

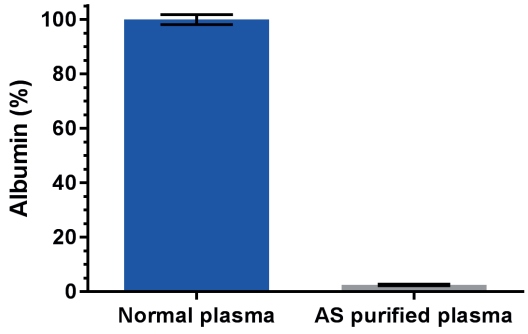

Figure 2: Level of albumin remaining without pretreatment (normal plasma) and with AS pretreatment (AS purified plasma), error bars represent SD with n=3

AS concentration and DNX recovery

This test was performed to determine the concentration AS needed that provides the highest recovery. Increasing concentrations AS were tested starting from 33.3% going up to 44.4%. From the results obtained we see that at 37.5% AS the line started to bend reaching a plateau at an AS concentration of 41.2% (Fig. 3). This translates to 70 µL saturated AS per 100 µL solution consisting of 10 µL sample and 90 µL Tris (50mM, pH 8, 0.5% OG).

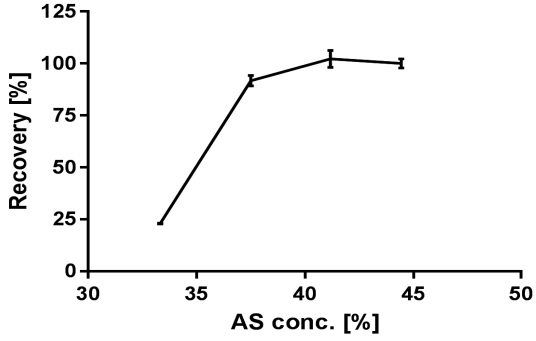

Figure 3: Concentration AS plotted against DNX recovery, error bars represent SD with n=3

Methanol concentration and DNX recovery

The second step in the procedure that could lead to loss of DNX is the water – methanol washing step. Water was used to dilute the SDS and methanol was used to precipitate the proteins in order to obtain a pellet after centrifugation. From the obtained results we see that the highest concentration methanol tested resulted in the lowest recovery (Fig. 4). This is counterintuitive since we expect the opposite. In fact, the reason for the signal drop is not due to loss of DNX in the washing step, but rather due to ionization suppression or trypsin denaturation caused by inefficient removal of SDS. Here, we notice that we need to introduce at least 50 µl of water per sample to dilute and remove SDS in the supernatant layer. Using 48% methanol, we noticed a decrease in DNX recovery and therefore, we chose 65% methanol as the optimum concentration which lies in the middle of the plateau.

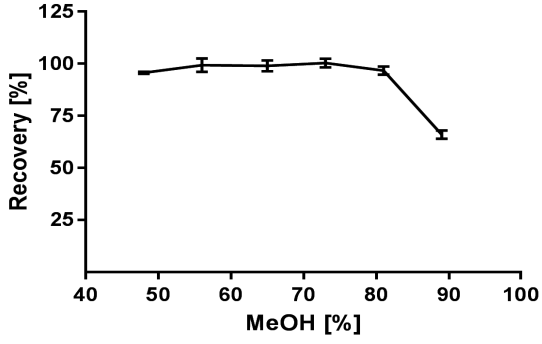

Figure 4: Methanol concentration plotted against DNX recovery, error bars represent SD with n=3.

This corresponds to 400µL methanol per 70µL sample diluted with 150µL water.

Comparison between DNX and DNX-β

DNX-β (CH14.18/CHO; Dinutuximab beta; Isquette) received marketing approval in 2017 and is currently used to treat new patients suffering from NB. DNX (CH14.18/SP2/0; Unituxin) was withdrawn from the market in March 2017. However, patients on Unituxin will continue to receive this formulation. DNX-β can be given without co-administration of interleukin-2, which induces inflammatory side effects [28]. These two drugs contain the same peptide sequences, only glycosylation differences can occur due to the different cell lines used. Since measurements are based on signal obtained from the signature peptide these two drugs were found to be well correlated with a bias of 8.8% and RSD of 1.13%. The latter illustrates one of the advantages of antibody quantification using LCMSMS.

Patient infusion data

One patient was monitored for DNX concentration with the above described method during the 4 day treatment period. Samples were drawn before infusion and at the end of a 10 to 20h DNX infusion. The third dose showed the highest DNX concentration (Fig. 5). This was probably due to relative short resting period (8h) in between dose 2 and 3. The resting period in between dose 1 and 2 and between dose 3 and 4 were 30 hours and 18 hours respectively.

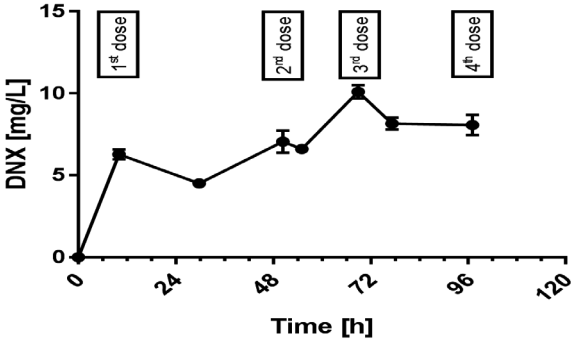

Figure 5: DNX concentration data obtained from one patient dosed during 4 days, each time with a 10 – 20 hour infusion of 14.01 mg DNX, error bars represent SD with n=2.

Validation

The LLOQ was first determined by analyzing DNX spiked in pooled normal human plasma sample at a concentration of 1 µg/mL (Fig. 6). The signal to noise ratio (S/N) was found to be approximately 187, which is well above the EMA guidelines (threshold of S/N >5).

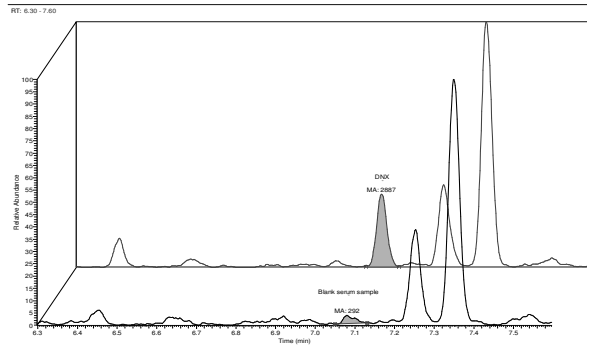

Figure 6: LLOQ at 1 µg/mL tested against a blank pooled normal human plasma sample

Linearity was evaluated during 3 days using the following standards; 1.00, 2.00, 4.00, 8.00, 16.00, 32.00 µg/mL. The back calculated concentrations were found to be in agreement with guidelines with a determination coefficient R^2>0.999 (Fig. 7).

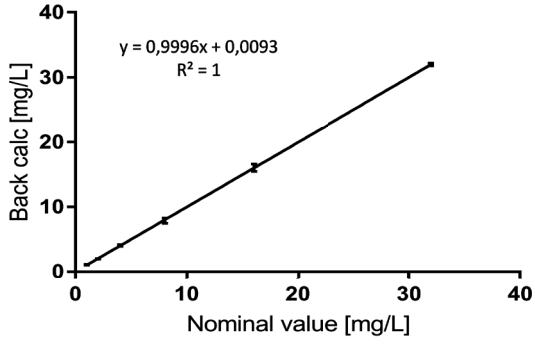

Figure 7: Linearity test, standard curve with SD error bars at each standard level tested during three days

Selectivity and carry-over were evaluated by analyzing 10 normal human plasma samples and measuring the signal intensity at the RT of the signature peptide in relation to LLOQ signal intensity. Table 3 lists the percentage signal in relation to LLOQ signal for DNX signature peptide and for the SIL IS.

Table 3. Selectivity and carry over test with randomly chosen blank human plasma samples.

Blank human plasma sample	% Signal in relation to LLOQ signal	% Signal in relation to IS signal
Plasma pool – IS (Carry over)	12	0.16
Plasma pool + IS	3	112.05
Sample 1	2	0.08
Sample 2	5	0.06
Sample 3	11	0.02
Sample 4	8	0.04
Sample 5	5	0.06
Sample 6	7	0.03
Sample 7	1	0.07
Sample 8	1	0.05
Sample 9	5	0.00
Sample 10	8	0.03

Matrix effect was tested by spiking DNX at QC Low and QC High levels in 7 different human plasma samples. The average bias was in concordance with EMA guidelines of 15% (table 4).

Table 4. Matrix effect test, 7 human plasma samples spiked at QC Low (3mg/L) and QC High level (25mg/L)

Sample nr.	Measured [mg/L]	Bias [%]	Measured [mg/L]	Bias [%]
1	2.80	-6,7	24,42	-2,3
2	2.60	-13,3	25,98	3,9
3	3.16	5,2	27,61	10,4
4	3.29	9,5	26,28	5,1
5	3.26	8,6	29,00	16,0
6	3.24	8,2	26,82	7,3
7	3.14	4,7	26,87	7,5

Auto sampler stability was tested by re-injecting the validation samples after 24 hours. Signal intensities and concentration values were found to be in agreement with previous run (data not show). Freeze and thaw stability was tested during 3 days with overall CV of around 4%

for both QC Low and QC High and a bias of 1.4% and 5%, respectively. And finally, within-run and between-run precision and accuracy for LLOQ, QC Low, QC Med and QC High were evaluated during three days in five folds and were found to be well within the acceptance criteria with CV in the range of 5% which is 3 times lower than the set limit see table 5.

Table 5. Accuracy and precision validation data for QC's at LLOQ, Low, Medium and High levels. 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	5.5	1.4	5.7	6.4
Low	4.4	1.2	4.6	0.2
Med	2.9	2.0	3.5	2.9
High	2.9	3.4	4.5	4.6

Conclusion and discussion

A novel sample work-up method utilizing AS in combination with SDS was developed for the quantification of total DNX in human plasma. The AS precipitation method facilitated efficient removal of albumin while retaining a high recovery for DNX. The development of a DNX LC-MS/MS method was performed because of the clinical need for reliable method to quantify DNX in plasma for ongoing pharmacokinetic studies and future drug monitoring. This method measures total (free and bound) DNX concentration in plasma while ELISA methods measure free DNX concentrations. Previous studies in animals and in humans showed that total mAb fraction measured by LC-MS/MS provide similar PK profiles as those obtained by ELISA's free mAb fraction [29-33]. Furthermore, Willrich and colleagues have shown that total infliximab was strongly correlated to free infliximab even in a subset of samples containing anti-drug antibodies [26].

These data show that similar results can be obtained with assays that are fundamentally different. In addition, LC-MS/MS methods are in many ways analytically superior to ligand binding assays due to their high specificity, wider linear dynamic range and higher accuracy and precision.

The method was validated in concordance with the latest EMA/FDA guidelines. Furthermore, excellent validation data were obtained. This was mainly due to the ease of use of the method and to the efficient and robust digestion process which was achieved by incorporating SDS in the sample work-up. Finally, the here described method can be used as a template for the quantification other therapeutic monoclonal antibodies in plasma.

Acknowledgements

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Endogenous protein quantification

3.1

Quantification of neutralizing anti-drug antibodies and their neutralizing capacity using competitive displacement and tandem mass spectrometry: Infliximab as proof of principle.

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Journal of Translational Autoimmunity, 1 (2019) 100004

Abstract

The development of anti-drug antibodies (ADA) in patients treated with therapeutic proteins can result in treatment failure. The clinically most relevant fraction of these antibodies are the neutralizing anti-drug antibodies (NAb) that block the pharmacological function of the drug. Consequently, the detection of NAb in plasma is a better predictor of loss of therapeutic response than increased levels of total anti-drug antibodies (ADA) test. Traditional assays to detect ADA and NAb have limited specificity, sensitivity and linear dynamic range.

Here, we demonstrate for the first time the potential of a LC-MS/MS method to measure the concentration of NAb against therapeutic proteins in plasma as exemplified with infliximab (IFX). We designed a competitive screening assay in which the presence of NAb in patients plasma prevents the binding of stable isotopically labeled (SIL) mAb infliximab to TNF-α ligand fixed on a 96-well plate.

After washing, eluting and digesting, the signal intensity of SIL IFX-derived signature peptides was inversely and strongly correlated with NAb concentration in the sample: R^2=0.999. Evaluation data showed that the assay has a high specificity (100%) and a high sensitivity (94%) to predict NAb presence. Cross-validation against total ADA measured by a reference laboratory using radio immunoassay assay (RIA) for ADA provided a good correlation (r^2 = 0.79).

We developed for the first time a robust and fast screening method on the basis of LC-MS/MS to determine the presence of NAb and its neutralizing capacity in plasma. The analyses of NAb can be combined with therapeutic mAb quantification. Furthermore, the quantification of the neutralizing capacity expressed as mAb mass equivalents opens the door to new personalized dosing strategies in patients with NAb.

Introduction

Therapeutic proteins are increasingly used as a treatment of human disease with over 3500 clinical trials ongoing worldwide [1]. Compared to small molecules, therapeutic proteins show a high risk of an autoimmune response, resulting in anti-drug antibodies (ADA) development, which is associated with treatment failure [2-4]. Steps have been undertaken to reduce the risk of immunogenicity such as the development of fully humanized forms of therapeutic monoclonal antibodies. However, immunogenicity regrettably remains a challenge in present-day clinical practice [5].

Two types of ADA can be distinguished, the neutralizing antibodies (NAb) and the non-neutralizing antibodies (non-NAb). NAb bind to the active site of the drug and inhibit its pharmacological function, while non-NAb bind to a site that is not involved in target binding and which renders the drug pharmacologically active, though its clearance from circulation can still be affected [6].

During the last decade, several clinical studies addressed the association of immunogenicity and clinical outcomes in patients using therapeutic proteins. For the tumour necrosis factor alpha (TNF-α) inhibitor infliximab (IFX) the results are contradictory. Some studies showed an association between ADA levels and loss of clinical response, while others could not confirm such an association [7, 8]. This discrepancy may be explained by the type of ADA assay used as studies focussing on NAb rather than on total ADA levels, are more likely to find a correlation with clinical loss of response [9, 10].

Traditionally, NAb levels are measured with cell-based assays, which quantify the biological activity of a living cell exposed to sample material [11, 12]. These assays are laborious to perform and may lack specificity due to cross reactivity, e.g. with cytokines or other unknown factors present in patient matrices, leading to false positive results [13]. Furthermore, standardization of cells-based assays can be challenging [14].

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has proven to be a reliable bioanalytical platform for both small and large molecule quantification [15-19], and offers various advantages over other techniques in terms of selectivity, linear dynamic range, method development time, enhanced precision due to the use of internal standards and multiplexing capabilities. Therefore, we evaluated for the first time the use of LC-MS/MS in combination with a selective purification to quantify the NAb to IFX (NATI) in human plasma.

Materials and methods

Chemicals and reagents

As a standard for the neutralizing antibodies to infliximab (NATI), a full length human anti-idiotypic antibody against IFX, clone AbD17841_hIgG1) was obtained as a 500 mg/L PBS solution from Bio-Rad laboratories (Hercules, CA, USA). Stable isotopically labeled (SIL) standard IFX and adalimumab (ADM) bio-similar were obtained from Promise advanced proteomics (Grenoble, France). Biotinylated human recombinant tumour necrosis factor alpha (b-TNF-α) was obtained from ACRO biosystems (Newark, DE, USA). Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Scientific (Waltham, MA, USA). TPCK-Trypsin was supplied by Thermo Scientific as a lyophilized powder and was dissolved in acetic acid (50mM) to a concentration of 10 µg/µL, aliquoted in Eppendorf LoBind Microcentrifuge tubes and stored at -80 °C. All other reagents and LC-MS grade mobile phase solvents were obtained from Sigma (Saint Louis, MO, USA).

Preparation of standards

Working standard at 96 mg/L NATI was prepared by diluting 500 mg/L stock solution in drug free pooled human plasma. Standards at concentrations of 1.5, 3, 6, 12, 24, 48, and 96 mg/L were freshly prepared from the working solutions by serial dilution in drug free pooled human plasma.

Instrumentation and chromatographic conditions

Sample purification was performed on a Vibramax 100 plate shaker, Heidolph Instruments (Schwabach, Germany). Sample denaturation and digestion was performed on Eppendorf ThermoMixer™ C (Hamburg, Germany). All measurements were performed on an Ultimate 3000 UHPLC Dionex (Sunnyvale, CA, USA) coupled to a TSQ Quantiva, Thermo Scientific (Waltham, MA, USA). The analytical column was Acclaim™, RSLC 120, C18, 2.1 x 100 mm, 2.2 µm particle size, Thermo Fisher. The guard column was the SecurityGuard column ULTRA C18, 2.1 mm Phenomenex (Torrence, CA, USA). Both columns were maintained at 50 °C. The mobile phases were: (a) 0.1 % formic acid (FA) in water; (b) 0.1 % FA in Acetonitrile (ACN). The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/5 (%

B), 3.00/35, 3.05/85, 3.95/85, 4.00/5 and 5.00/5. The flow rate was 0.6 mL/min and the run time was 5 min. The MS was operated in positive mode with spray voltage of 3.5 kV, Ion Transfer Tube Temperature 350 °C, vaporizer temperature 300 °C, aux gas pressure 15 Arb, sheath gas pressure 50 Arb, sweep gas pressure 0 Arb and collision gas pressure 2.5 mTorr. The precursor ions, product ions, collision energy and radio frequency (RF) lens settings are listed in Table 1 for SIL IFX and SIL adalimumab (ADM) which was used as an internal standard.

Table 1. TSQ Quantiva SRM transitions and settings for the signature tryptic peptides of ADM, IFX and SIL internal standard (IS).

Peptide sequence	Analyte	Used as	Precursor [m/z]	Product [m/z]	Product ion	Charge	CE[a] [V]	RF[b] [V]
APYTFGQGTK[13C6,15N2]	ADM	IS	539.27	746.38	y7	1+	20	80
DILLTQSPAILSVSP-GER[13C6,15N4]	IFX	Quantifier	953.53	555.25	y5	1+	25	110
YASESMSGIPSR[13C6,15N4]	IFX	Qualifier	647.80	844.42	y8	1+	25	90
ASQFVGSSIH-WYQQR[13C6,15N4]	IFX	Qualifier	601.96	759.38	y12	2+	15	80

[a] CE: Collision energy

[b] RF: Radio frequency lens

Sample preparation

Sample preparation was based on an immunoaffinity LC-MS/MS method for the simultaneous quantification of ADM and IFX, previously published by our group [19]. Figure 1 displays the general principle of the method. The presence of NATI results in a reduction of SIL IFX signature peptide signal.

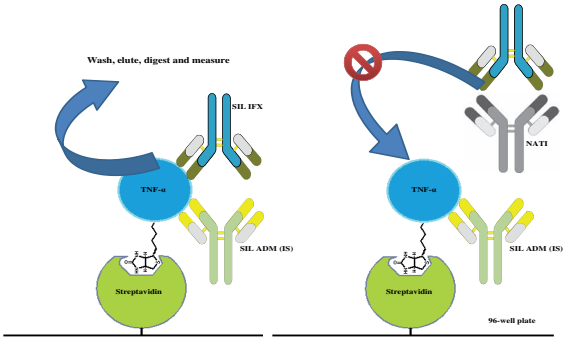

Figure 1: Principle of the assay; In the absence of NATI in the sample, the maximum signal is measured (left). Lower SIL IFX signal is obtained proportional to the concentration NATI present in the sample (right). SIL ADM is used as internal standard to correct for loss during sample preparation and as a quality assurance indicator.

Ten times molar excess b-TNF-α was captured on a streptavidin coated 96 well plate by adding 20 µL b-TNF-α (50 µg/mL) to 180 µL PBS (0.1% Tween-20) in each well and allowed to bind for 3 hours on a plate shaker (500 rpm) at room temperature. After binding b-TNF-α to the plate, the excess unbound b-TNF-α was washed away with three times 200 µL PBS (0.1% Tween-20). Thereafter, in a LoBind 1.5 mL Eppendorf tube, 190 µL PBS (0.1% Tween-20) was added followed by 5 µL of standard, QC or patient's plasma sample and 5µL mixed working solution consisting of SIL ADM and SIL IFX (10 µg/mL) and gently vortexed for 5 minutes. In this step, the NATI present in the standard, QC or sample binds to the SIL IFX thus neutralizing its binding epitopes. An effect on SIL ADM is unlikely since there are no antibodies present that can neutralize it.

The mixture was transferred to the b-TNF-α pre-coated 96-well plate and allowed to bind for 3 hour at room temperature on a plate shaker (500 rpm). Here, the SIL ADM was free to bind to b-TNF-α, in contrast to NATI conjugated SIL IFX. Next, the wells were washed three times with 200 µL PBS (0.1 % Tween-20) to remove the unbound conjugate NATI-SIL IFX. The retained SIL IFX and SIL ADM were eluted with 100 µL 0.1% FA 0.5% octyl glucoside by vortex mixing for 5 minutes at 1200 rpm. Extracts were transferred to a 500 µL 96-well plate and heat denatured on a ThermoMixer C block heater set at 80 °C for 30 minutes to unfold the protein and allow for efficient digestion. Samples were centrifuged at 4000g for 5 minutes and 5 µL trypsin (1 µg/µL) was added and gently mixed. Then, the plate was placed in the ThermoMixer C block heater set at 37 °C for overnight digestion. Finally, trypsin activity was stopped through the addition of 20 µL 10 % FA in ACN and 25 µL was injected and analyzed on LC-MS/MS. Here, the mass spectrometer measured the signal intensity of unique signature peptides derived from SIL ADM and SIL IFX which was directly correlated with concentration (Table 1).

Method evaluation

A calibration curve was set up covering a range of 0, 1.5, 3, 6, 12, 24, 48, 96 µg/mL NATI and was plotted in logarithmic scale on the x-axis using four-parameter logistic regression versus the signal ratio SIL IFX/IS. Calculations and figures were made with GraphPad Prism version 8.0.1.

As this method was developed as a screening method a partial validation directed at the relevant screening parameters was performed. Method's performance criteria such as critical limit (Lc), detection limit (Ld), matrix effect, specificity and sensitivity were evaluated.

Critical limit was set at a standard deviation at 1.65 times the blank plasma back calculated concentration leading to an α error of 5% (probability of false positives). Detection limit was set at the standard deviation × 3.3, which provides an α error of 0.05%. Matrix effect, specificity and sensitivity was tested by measuring 6 blank human plasma samples from individual volunteers and the same 6 blank plasma samples spiked at 2 µg/mL NATI. These samples were aliquoted and stored at -80 °C and measured in six fold during three different days to determine the reproducibility and repeatability.

RIA method for binding antibodies

The RIA reference method used in this study measures total ADA to IFX in plasma and was developed by Sanquin (Amsterdam, The Netherlands) [20].

Clinical samples

Remnant plasma sample material of patients, who were treated at the University Medical Center Utrecht (UMCU, The Netherlands) for rheumatoid arthritis and inflammatory bowel disease were used with patients' consent. Aliquots were sent for total ADA quantification by RIA analysis at Sanquin (Amsterdam, The Netherlands) and the remainder was stored at -80 °C before LC-MS/MS analysis. Forty samples with IFX levels <1 µg/mL were chosen for NATI analysis, covering a total ADA range from 0 to >880 AU/mL (Arbitrary Units). Thirty two samples where negative for total ADA and did not contain detectable IFX concentration these samples were used for critical and detection limit assessment.

Results and discussion

Method development

During routine analysis of free IFX and free ADM concentration in plasma samples with our previously developed LC-MS/MS assay, we observed that some patients' samples with an undetectable free IFX concentration showed a diminished SIL IFX internal standard signal. [19] We hypothesized that this was caused by the presence of NATI in the patient samples (Fig. 1). Indeed, in contrast to physiological TNF-α, which is only present in low concentrations

(80 to 300 pg/mL) in plasma, [21] NATI can be present in high enough concentrations to block the binding epitopes of SIL IFX solution (10µg/mL) thus preventing it from binding to b-TNF-α coated streptavidin plate. [22] Non-neutralizing antibodies are also able to bind to SIL IFX, however the active epitopes of SIL IFX remain free to bind to b-TNF-α and can thus be captured for measurement. Furthermore, the samples that showed a reduced SIL IFX signal, were also sent to a reference laboratory for ADA measurements and all were found to be positive for ADA. Spiking experiments of blank samples with commercially obtained NATI were performed to reproduce the signal reduction. Addition of 5 µL of the serially diluted (96, 48, 24, 12, 6, 3, 1.5 and 0 µg/mL) NATI to a fixed amount 5 µL of 10 µg/mL (50 ng) of SIL IFX resulted in a calibration curve with good correlation (R^2= 0.999) using a four-parameter logistic regression model, thus confirming our hypothesis (Fig. 2). The SIL ADM which was used in the original method as an internal standard for ADM quantification, was found to be suitable as an internal standard for NATI determination. Here, SIL ADM provided a means to correct for component loss incurred during sample preparation such as binding, washing and eluting steps, thus enhancing assay precision and accuracy. SIL ADM signal intensity showed minimal variability even with elevated NATI concentrations. Furthermore, to ensure that SIL ADM did not interfere with SIL IFX binding, 10 times molar excess b-TNF-α was used in relation to both SIL IFX and ADM as was established in our previous work [19].

Method evaluation

The calibration curve which had a range from 0 – 96 µg/mL NATI provided an excellent coefficient and determination (R2=0.999).

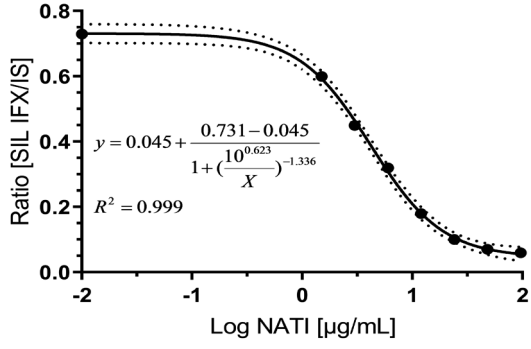

Figure 2: Four-parameter logistic regression function was used for the NATI calibration curve. X-axis range (0 – 96 µg/mL) was converted to logarithmic scale, y-axis represents ratio IFX signal divided by IS (SIL AMD) signal.

At 0 µg/mL NATI, the 5µL mixture of working solution which consisted of SIL ADM and SIL IFX (10 µg/mL) was free to bind to the b-TNF-α coated streptavidin plate and maximum signal was obtained after elution, denaturation, digestion and measurement for their signature peptides (Fig. 2). With increasing concentrations of NATI, a decreased signal was observed for SIL IFX signature peptides, providing an excellent inverse correlation. SIL ADM signal remained stable regardless of NATI concentration and was used as internal standard. The critical limit (Lc) and detection limit (Ld) were assessed using 32 samples with an undetectable NATI concentration (Fig. 3).

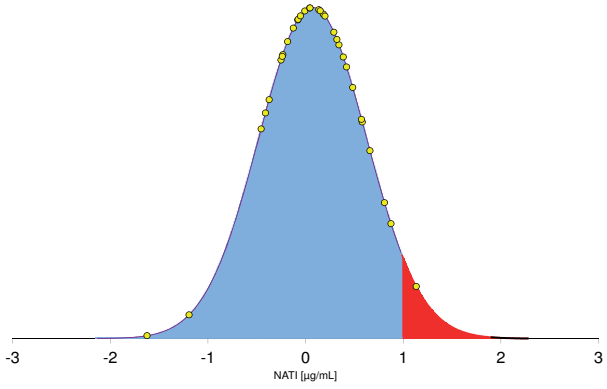

Figure 3: Normal distribution of samples (n= 32) with no NATI, red border at 0.98 µg/mL represents Lc, black border at 1.89 represents Ld

A critical limit and a detection limit of 0.98 µg/mL and 1.89 µg/mL, respectively, were defined. Therefore, these limits were set at 1 and 2 µg/mL, respectively and tested further by analysing plasma samples from volunteers unexposed to infliximab.

Levels of NATI in these samples were under the detection limit and no false positives occurred (Fig. 4). The critical level was tested by spiking the same blank samples with 2 µg/mL anti-idiotypic antibody. Here, only one out of 18 samples was below the critical limit, thus providing a sensitivity of 94.4 % (Fig.4).

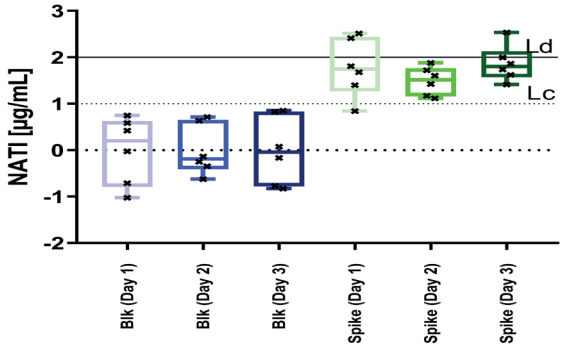

Figure 4: Box and whiskers plot of the critical (Lc) and detection (Ld) levels. Data obtained from six individual human plasma samples never treated with infliximab (blk) and the same samples spiked with NATI (Spike). Test performed on 3 different days.

Comparison of ADA and NATI results

Prior to sample analysis, the conversion factor for the Bio-Rad anti-idiotypic antibody standard was determined. Here, 1 µg/mL standard with a monovalent intrinsic affinity of KD = 1.8 nM was found to be equivalent to 10 AU/mL measured by RIA (Sanquin). Samples were prepared and analyzed including a calibration curve ranging from 0 – 96 µg/mL. The results showed a clear association between total ADA and NATI concentrations in human plasma sample (Fig. 5).

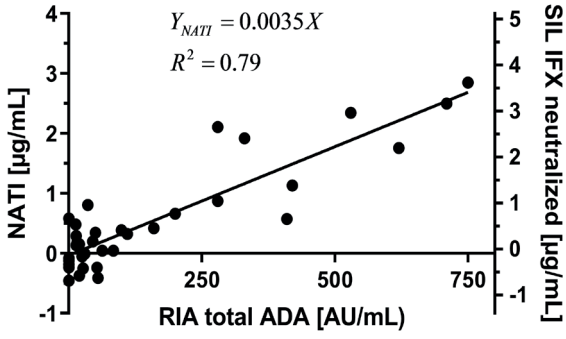

Figure 5: Total ADA versus NATI (lefty-axes) and SIL IFX neutralized (right y-axes), n = 36, levels >880 AU/mL were not included

Surprisingly, the conversion of NATI concentration (µg/mL) to AU/mL values indicated that 3.5% of total ADA are of the neutralizing type. However, this value could be an underestimation of the true concentration, since the standard used for calculation had a high binding affinity to infliximab. This low percentage could also be explained by the fact that the chimeric monoclonal antibody infliximab contains multiple mouse framework regions in the variable heavy and light chains that are not involved in target binding but can induce an immune response. These mouse framework regions would result in binding antibodies being formed, but not necessarily neutralizing antibodies.

Most of the ADA positive samples tested were within the first three standard point of the NATI calibration curve (0-3.0 µg/mL), which is equivalent to a neutralizing capacity of 0-3.8µg IFX/mL plasma. Five samples were excluded from the comparison due to the upper quantification limit of the reference RIA assay (>880 AU/mL). These samples ranged between 3-96 µg/mL NATI.

Discussion and conclusion

Here, we describe for the first time the use of LC-MS/MS for the determination of NATI in human plasma samples. The screenings assay was highly selective (100%) and sensitive (94%) for the detection of NATI presence in plasma. Cross validation against RIA total ADA resulted in a good agreement between methods. By implementing the sample preparation method used for the simultaneous quantification of free ADM and IFX, multicomponent analysis of IFX, ADM together with NATI was achieved. Here, ADM and IFX plasma samples can be batched and analyzed together thus enhancing sample throughput. Moreover, NATI presence can also be identified when sub therapeutic levels (<1µg/mL) of IFX are detected. This is highly advantageous over commercial ELISA assays since these only allow for one component analysis per test. Importantly, the measurement of ADA with RIA is less informative for loss of response since the formation of non-neutralizing antibodies occurs at an early stage and can be transient. Indeed, cases have been reported where patients developed immune tolerance to the therapeutic protein which has led to the disappearance of ADA over time [23]. However, through further expansion and diversification of the B cell population, NAb can be formed at a later stage and are more likely to persist [24]. Unlike total ADA measurement which in some cases only consist of non-neutralizing antibodies, the here presented LC-MS/MS method measures only NAb fraction and can thus be more meaningful to clinicians in clinical decision making.

Physiological TNF-α in these patients is usually elevated, nevertheless free circulating TNF-α only ranges between 80 to 300 pg/mL in plasma and will thus only have a marginal contribution to the neutralization of SIL IFX and SIL ADM which are present in μg/mL [21]. Furthermore, as was demonstrated in our previous work [19], the incorporation of molar excess TNF-α (1μg) per well, provided excellent recovery for both components.

The principle described here can be used as a template to detect NAbs to therapeutic proteins using LC-MS/MS. The screening assay proved to be robust, fast and can be multiplexed with quantification of drug levels. Finally, the absolute quantification of the neutralizing capacity translated to therapeutic protein mass equivalents may potentially open the door for new personalized dosing strategies in patients with NAb. By dosing molar excess drug in relation to NAb present, a viable drug titer might be achieved in plasma thus counteracting loss of response to treatment with therapeutic proteins.

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3.2

Quantification of coagulation factor VIII in human plasma with liquid chromatography tandem mass spectrometry using a selective sample purification with camelid nanobodies

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Abstract

Patients with hemophilia A are currently diagnosed and monitored by measuring the activity of coagulation factor VIII (FVIII) in plasma mostly with the one-stage clotting assay (OSA). Although the OSA is routinely available in many clinical laboratories, it has in some circumstances relatively low sensitivity and specificity. Therefore, the FVIII activity as a biomarker does not always correlate with the bleeding phenotype. Therefore, we have developed a liquid chromatography tandem mass spectrometry method to quantify the concentration of coagulation FVIII in plasma which would allow us to investigate the relation between FVIII plasma concentration, FVIII activity and bleeding tendency in future studies.

LC-MS/MS method was set up by firstly dissociation Von Willebrand factor (VWF) from coagulation factor VIII by triggering the coagulation cascade to occur thus generating active factor VIII (FVIIIa). FVIIIa was then selectively extracted by means of immunoaffinity interaction using anti-FVIII camelid nanobody, after which FVIIIa was eluted, heat denatured and trypsin digested. Finally, a FVIII specific peptide was used as a surrogate for quantification by mass spectrometry. Critical method parameters such as antibody amount, incubation time, sample volume and type of streptavidin 96 well plate were optimized.

The method was validated according to European Medicines Agency (EMA) guidelines where an LLOQ of 1 ng/mL was obtained using 50 µL of citrate plasma sample. Within-run and between-run accuracy and precision for quality control (QC) samples, LLOQ (1 ng/mL), QC Low (5 ng/mL), QC Med (150 ng/mL), QC High (300 ng/mL) were within the threshold of 15% relative standard deviation (RSD) and Bias.

The selective immunoaffinity method which was used in combination with a highly sensitive mass spectrometer allowed for an unpresented LLOQ of 1 ng/mL utilizing 50 µL plasma sample. This method will be used to investigate the beneficial value of FVIII plasma concentration which may be used in conjunction with FVIII activity for patient diagnosis and dosage optimization.

Introduction

Congenital hemophilia A is a clotting disease caused by a defect in the Factor VIII (FVIII) gene located on the X-chromosome and therefore predominantly affects the male population. The prevalence of hemophilia A is 1 in 5.000 male persons. Hemophilia A can lead to serious complications such as bleeding after surgery, bruising, disabling arthropathy, gastrointestinal and urological bleeding and intracranial hemorrhage [1-5]. Acquired hemophilia A is rare and has been estimated to affect 0.2 – 1 in 1 million persons per year [4]. Acquired hemophilia A is caused by the development of auto-antibodies directed against FVIII [4, 6-8]. The majority of the patient population (60%), which consists predominantly of men around the age of 60 years, have a severe type of hemophilia A with FVIII activity usually below 3%. The current treatment requires infusion with recombinant or plasma derived FVIII [9]. Furthermore, Patients with congenital hemophilia are also at risk of developing inhibitors against FVIII. Some of these patients also require high dose FVIIa, which may increase the risk of thrombosis. Patients with either congenital or acquired hemophilia A are diagnosed and treated depending on the activity of FVIII in plasma and therefore careful monitoring of these patients is critical [10].

Currently, there are three commercially available assay types for measuring FVIII activity: a one-stage assay, a two-stage assay, and a chromogenic (Amidolytic) assay [11-14]. The most commonly used assay is the one-stage assay, which is based on activated partial thromboplastin time (aPTT) [15, 16]. This assay measures the time required for patient plasma to form a fibrin clot after it has been combined with a FVIII deficient plasma and reagents to initiate clotting have been added [17, 18]. The one-stage assay is relatively fast and cheap to perform on automated coagulation analyzers. However, this assay has been shown to have higher %CV for elevated FVIII values in comparison to the other methods [14, 19]. Depending on the endpoint detection method, the one stage-assay may be more sensitive for lipemic plasma samples, and the assay may also have varying sensitivity and specificity to anticoagulant medication and to endogenous inhibitors such as antibodies against FVIII or lupus anticoagulant. Depending on the dilution factor and the activator used in the one-stage assay, both of these types of antibodies may lead to a prolonged aPTT value, which would result in a low FVIII activity, hence complicating diagnosis [20, 21]. Furthermore, due to the lack of standardization in instrumentations and reagents the intra-laboratory variation is the highest in this type of assay [11]. Finally, some patients with mild or moderate hemophilia A will show correct lower activity in the chromogenic assay and two-stage assay but will show false normal activity with the one stage assay possibly leading to misdiagnosis [11, 22].

Aside from these technical limitations in the one-stage assay, activity-based assays in general are sensitive to sample condition. Citrate plasma kept at room temperature (25 °C) or

at 4 °C needs to be analyzed within 2 to 6 hours after sample collection, due to the rapid degradation of FVIII, alternatively, the samples can be quickly processed and frozen at -80°C [23-27]. In contrast to LC-MS/MS methods, ligand binding assays pose other challenges such as cross reactivity and limited linearity. Therefore, in order to eliminate the drawbacks mentioned above, we have investigated the suitability of liquid chromatography tandem mass spectrometry (LC-MS/MS) in the measurement of FVIII concentration in plasma. The method proposed is based on immunoaffinity purification in combination with tryptic digestion and LC-MS/MS analysis.

Potentially, LC-MS/MS may be complementary to the current activity-based assay due to its ability to measure the absolute plasma concentration. Furthermore, pharmacokinetic and pharmacodynamics studies can be performed with FVIII plasma concentration which might be a better predictor for the bleeding phenotype compared to the current activity based assay. In addition, the stability issue of plasma samples, which has been proven to influence the activity results, could be circumvented by measuring FVIII concentration. This could bring home monitoring for hemophiliacs one step closer by allowing mail-in samples. Finally, other coagulation factors can also be added to the existing method at a later stage thus allowing for multiplexed LC-MS/MS analysis.

Materials and methods

Chemicals and reagents

Octocog alfa (Advate™) was obtained from Baxter (Lessines, Belgium) as lyophilized powder and was reconstituted in LC-MS grade water to a final concentration of 500 IU/mL equivalent to 94 µg/mL FVIII; 40 µL aliquots of this solution were pipetted in Eppendorf LoBind™ Microcentrifuge tubes and stored at -80 °C. Stable isotope labeled peptide internal standard (IS) GELNEHLGLLGPYIR[$^{13}C_6$, $^{15}N_4$] was synthesized by Pepscan (Lelystad, The Netherlands) as a 1 mg lyophilized powder and was dissolved in 1 mL elution solvent (0.5% trifluoroacetic acid (TFA) in 50 % methanol, 50% water). Biotinylated Anti FVIII conjugate, reference number 7102862100 was obtained from Thermoscientific (Waltham, MA, USA) as a 1 mg/mL solution and was stored in -20 °C. Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Fisher (Waltham, MA, USA). MS grade modified trypsin was obtained from Promega (Madison, WI, USA) and was dissolved to 0.1 µg/µL in 50 mM acetic acid and aliquoted in Eppendorf LoBind™ microcentrifuge tubes. Aliquots were stored at -80 °C. FVIII deficient human plasma was obtained from Precision BioLogic Inc.

(Dartmouth, NS, Canada). All other reagents and LC-MS grade mobile phase solvents were obtained from Sigma (Saint Louis, MO, USA).

Preparation of standards, Internal standard and QCs

The FVIII working solution (500 ng/mL) was prepared fresh from 94000 ng/mL stock solution by diluting in FVIII deficient human plasma. Standards at concentrations of 500, 200, 80, 40, 16, 4 and 1 ng/mL were prepared from the working solution by serial dilution in FVIII deficient human plasma. Before use, the IS solution (1 µg/µL) was diluted to 5 ng/mL in 0.1% formic acid (FA) and 0.005% Zwittergent™ 3-16. Quality Control samples (QCs) where prepared at 4 levels namely; at lower limit of quantification (LLOQ) (1 ng/mL), QC low (5 ng/mL), QC med (150 ng/mL) and QC high (300 ng/mL). Aliquots were stored at -80 °C.

Instrumentation and chromatographic conditions

Sample purification was performed on a vibramax 100 plate shaker (Heidolph Instruments, Schwabach, Germany). Sample digestion was performed on a ThermoMixer, Eppendorf (Nijmegen, The Netherlands). All experiments were performed on an Vanquish UHPLC coupled to a TSQ Altis, Thermo Fisher (Waltham, MA, USA). The analytical column was Acclaim™, RSLC 120, C18, 2.1 x 100 mm, 2.2 µm particle size obtained from Thermo Fisher and was maintained at 50 °C. The mobile phases were: (a) 0.1 % formic acid in water; (b) 0.1 % formic acid in ACN. The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/10 (% B), 8/25, 8.1/80, 10/80, 10.1/10 and 12/10. The flow rate was 0.6 mL/min and the run time was 9 min.

Table 1. TSQ Altis Mass Spectrometry conditions for SRM transitions for the signature peptide liberated from FVIII after digestion with trypsin and the internal standard (IS) stable isotopic labelled FVIII peptide.

Peptide sequence	RT[a] [min]	Precursor Charge	Precursor [m/z]	Product Charge	Product [m/z]	Product ion	Dwell time (ms)
GELNEHLGLLGPYIR	7.2	3+	560.97	2+	747.93	Y5	340
GELNEHLGLLGPYIR[13C6,15N4], (IS)	7.2	3+	564.31	2+	752.93	Y5	60

[a]RT: Retention time

[b]CE: Collision energy

[c]RF: Radio frequency lens

The MS was operated in positive mode with spray voltage of 2.5 kV, Ion Transfer Tube Temperature 400 °C, vaporizer temperature 350 °C, aux gas pressure 20 Arb, sheath gas pressure 40 Arb, sweep gas pressure 0 Arb, collision gas pressure 2.5 mTorr, Collision Energy 16 and radio frequency lens 65. The precursor ions, product ions, retention and dwell time settings are listed in Table 1 for FVIII signature peptide and for the stable isotopic labeled internal standard.

Sample preparation

Sample preparation was based on immunoaffinity purification where the light chain of FVIIIa was captured by means of a biotinylated camelid nanobody (b-anti-FVIII) which in turn was bound to a streptavidin coated 96 well plate (Fig. 1).

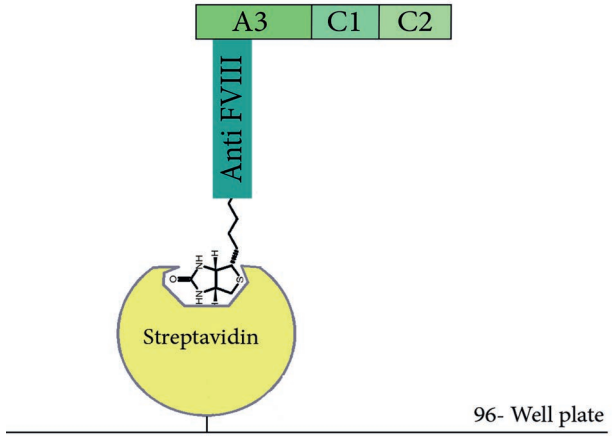

Figure 1. Sample purification of FVIII in plasma. The light chain of active factor FVIII is captured by means of immunoaffinity interaction using anti FVIII camelid nanobody fixed onto a 96 well plate by means of biotin-streptavidin interaction.

In day 1, b-anti-FVIII was coupled to a streptavidin coated 96 well plate by pipetting 200 µL b-anti-FVIII (1 ng/µL) dissolved in PBS, 0.05% Tween-20, 0.1% BSA in each well, followed by 3 hour binding on a plate shaker (300 rpm) at room temperature. The plate was washed 3 times with 200 µL PBS (0.1% Tween-20, 0.1% BSA) and stored upside down in a zip lock bag at-80 °C.

On day 2, 100 µL PBS (0.05% Tween-20, 0.1% BSA) and 50 µL sample (standard or QC) were pipetted to the antibody coated 96 well plate. Then, vWF was dissociated from FVIII by

adding 2.5 µL (Ca 2.5M + Thrombin 500 IE/mL) followed by an overnight incubation at 400 rpm on a plate shaker.
The next day, the wells were washed three times with 200 µL PBS (0.05% Tween-20, 0.1% BSA). Then, 100 µL IS solution (5 ng/mL) in elution solvent (0.1% FA with 0.005% Zwittergent™ 3-16 dissolved in water) was added to each well and mixed for 5 min at 1200 rpm on a plate shaker. The sample extracts were transferred to a 500 µL LoBind eppendorf 96-well plate and heat denatured at 80°C for 60 min. The samples were neutralized with 10 µL Tris (1M). Then, 5 µL trypsin (0.1 µg/µL) was added to each well and the sample plate was placed in a ThermoMixer set at 37 °C for 3 hour digestion at 800 RPM. Trypsin activity was stopped by adding 20 µL 10% formic acid dissolved in 100 % acetonitrile and finally, 50 µL was injected on a LC-MS/MS.

Camelid nanobody amount and signature peptide signal intensity

This experiment was performed to determine the amount of biotinylated camelid nanobody required to retain 75µL of the highest standard (500ng/mL) Octocog alfa in FVIII deficient plasma. The plate was coated with 10, 40, 100, 250 and 500 ng antibody per well each in triplicate. The highest standard was purified with a 4 hours incubation time and analyzed.

Binding time and signature peptide signal intensity

Citrate plasma sample from a volunteer was used to determine the time needed for the dissociation of vWF, conversion of FVIII to FVIIIa and for optimum sample binding during immunoaffinity interaction. Incubation times of 1, 2, 3, 4, 5 and 24 were evaluated in triplicate.

Effect of sample volume on signal intensity

Variable sample volumes were evaluated to determine the matrix effect on sample recovery. Citrated plasma from a volunteer was used for this test. The procedure described in sample preparation section was used with variable sample volumes (5, 10, 25 and 50 µL; each in duplicate).

Streptavidin 96 well plate brand and capacity test

Two high capacity streptavidin 96 well plates were compared. One plate was obtained from Sigma-Aldrich (SigmaScreen) and the other from Thermo Scientific (Streptavidin Coated High Capacity Plates). The test was performed according to the procedure described above using 25, 50 and 75 µL standards (500 ng/mL) in duplicate on both plates.

Validation of FVIII LC-MS/MS method

The validation was performed according to EMA guidelines which requires the evaluation of LLOQ, linearity, accuracy and precision, carry-over, auto sampler stability, freeze and thaw stability and matrix effect [28]. The acceptance criterion for LLOQ was that the signal of the QC LLOQ level (1 ng/mL) should be at least 5x that of the blank sample which consisted of FVIII deficient human citrated plasma. The calibration curve used to establish linearity consisted of 7 standards ranging from 1 to 500 ng/mL and was analyzed on 3 separate days. Within run and between run accuracy expressed as %bias and precision expressed as %CV were validated by measuring four QC levels (LLOQ (1 ng/mL), QC low (5 ng/mL), QC med (150 ng/mL) and QC high (300 ng/mL)) in five-fold during three days. The overall bias was calculated from the mean concentration and the within-run and between-run %CV was calculated from one-way ANOVA derived mean squares. Carry-over effect was tested by injecting a blank sample after the highest standard and comparing the signal intensity at the analyte retention time to the signal intensity of the LLOQ. Auto-sampler stability was evaluated by re-injecting the samples on the next day. Overnight stability was evaluated on QC low and QC high sample. Matrix effect was evaluated by spiking random samples with FVIII and calculating the spike recovery.

Results and discussion

Method Development

Human FVIII consists of a light chain and a heavy chain held together by calcium ion and by the stabilizing protein von Willebrand factor that is bound to the light chain of FVIII. The antibody used for sample purification targets the light chain of FVIII and therefore dissociation of von Willebrand factor was necessary to obtain a high recovery. This was achieved by triggering the coagulation cascade to occur through the addition of calcium chloride and thrombin to the citrated plasma sample. The amount of antibody used per well to capture FVIII was 200

ng providing around 100× molar excess in relation to the highest standard used. This was based on previous work with similar interaction where a therapeutic antibody was purified by means of a ligand [29]. However, FVIII concentration range in plasma is around 100× lower compared to therapeutic monoclonal antibodies, and therefore, the selection of the signature peptide was primarily based on the peptide that had low background interference and delivered the highest signal to noise ratio to meet assay requirements. The peptide 'GELNEHLGLLGPYIR' was found to meet this criterion and was found to be unique to FVIII. A stable isotopic labeled peptide GELNEHLGLLGPYIR[$13C_6$, $15N_4$] was synthesized and was used as internal standard to correct for MS ionization variability. Sample handling procedure was based on our previous work where we have compared various denaturation conditions for simultaneous quantification of adalimumab and infliximab [30]. Using the 80°C denaturation procedure we found that most signature peptides provided similar digestion efficiency as the commercial Smart Digest kit and was superior to denaturation and reduction with DTT at 60°C. Finally, remaining steps in the method were evaluated and optimized to ensure a sensitive, repeatable and accurate result.

Camelid nanobody amount and signature peptide signal intensity

The optimum amount of biotinylated camelid nanobody needed for efficient purification of activated FVIII light chain was investigated.

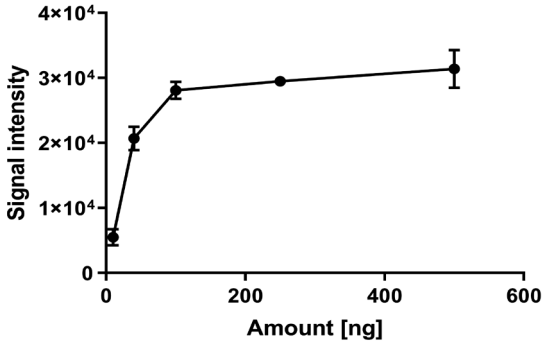

Figure 2. On the x-axis, coated amount of biotinylated camelid anti FVIII nanobodies are plotted versus signal intensity of the signature peptide of the highest standard (500ng/µL) on the y-axis, error bars represent SD with n=3

Camelid nanobody consist of an antibody variable domain fragment with a molecular mass

of 13kDa targeting the light chain of FVIII. This is an important advantage over traditional full length antibodies derived from other mammals which are 10 times bigger. More camelid nanobody can fit in each streptavidin coated well thus providing increased binding sites for factor FVIII. The saturation curve in figure 2 showed there was no significant (p=0.075) change in signal intensity between 100 and 500ng camelid nanobody. Therefore, 200 ng (15 pmol) camelid nanobody per well was used for all experimentations.

Binding time and signature peptide signal intensity

Another important parameter is the time required for the dissociation of vWF, the conversion of FVIII to FVIIIa and the binding of FVIIIa light chain to the 96 well plate coated camelid nanobodies. This experiment was performed with human derived FVIII plasma, since octocog alfa in the standard solution is not pre-conjugated with VWF. At 24 hours the signal intensity was significantly higher (p=0.0004) than the signal intensity obtained at 5 hours (Fig. 3).

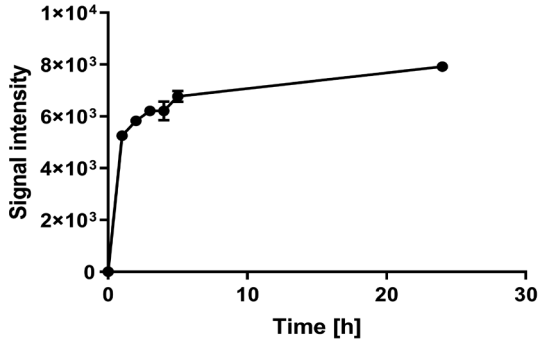

Figure 3. On the x-axis binding time between camelid nanobodies and the light chain fragment of FVIII is plotted versus signature peptide signal intensity from a volunteer sample on the y-axis, error bars represent mean with SD, n=3.

However, the difference in signal intensity between 1 and 24 hours was only 30%. Since the signal intensity at 24 hour was significantly higher than 5 hours, an incubation period of 24 hours was chosen. An even longer incubation period than 24 hour was not deemed necessary since the difference in signal intensity between 5 and 24 hours was only 15%.

Effect of sample volume on signal intensity

This experiment was set-up to determine if sample matrix interferes with FVIII binding or LC-MS/MS signature peptide measurement. The signal intensity obtained after sample purification and measurement was corrected for sample volume.

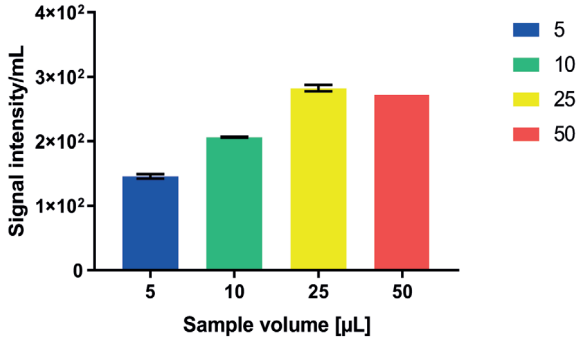

Figure 4. On the x-axis sample volumes from one volunteer is plotted versus the signal intensity corrected for volume on the y-axis; error bars represent mean with SD, n=2

Figure 4 shows that volume 50 and 25µL produced similar (p=0.051) signal intensities when corrected for volume. However, when sample volume lower than 25µL was purified, lower signal intensity was obtained than expected. Internal standard signal was stable for all samples indicating that no ionization differences was present due to matrix. A possible reason for the diminished signal intensity could be due to other coagulation factors being present in lower levels which might have affected FVIII activation. FVIIIa light chain is smaller and can easily be captured compared to intact FVIII. Since a low detection level were required, 50 µL sample was used. The fibrin blood clot obtained after overnight incubation was too big with higher >50µL sample volumes. This made sample handling such as decanting and washing difficult to perform.

96 well plate brand and capacity test

Streptavidin and biotin interactions are amongst strongest biological interactions known with binding strengths equivalent to covalent bonds. Furthermore, biotin and streptavidin bind selectively with each other, thus limiting cross-reactivity. Even though the Sigma plate had the highest capacity (>300 pmol/well) compared to the Thermo Scientific ~125pmol/well, the results show that both plates performed equally well (Fig. 5).

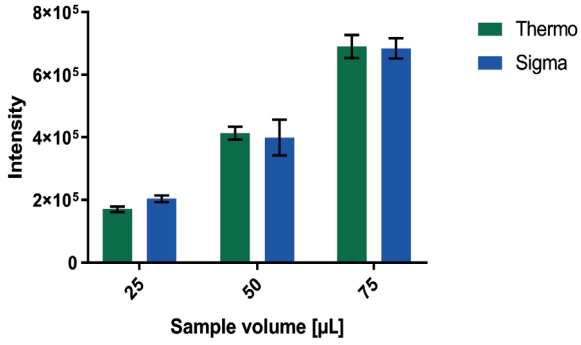

Figure 5. Signal intensity obtained with different streptavidin coated 96 well plates, namely the SigmaScreen from Sigma and Thermo Scientific Pierce Streptavidin Coated High Capacity Plates using various sample volumes of the highest standard 500ng/mL, errors bars representing SD with n=2.

The linearity was maintained even at the highest volume of 75µL (500 ng/mL) and the variations in duplicates were similar between plates and low. The reason for this good agreement is because only 0.1 pmol FVIII needed to be purified in the highest standard and both plates had more than 1000× molar access streptavidin per FVIII available. Thermo Scientific plates were chosen because they were significantly cheaper and were available in 8-well strip format.

Validation

First, a test was performed to determine whether the LLOQ level of 1 ng/mL was achievable. After performing the sample purification as described above, LLOQ sample was analyzed together with a blank (FVIII deficient plasma). The signal to noise (S/N) ratio at the retention time of the surrogate peptide "GELNEHLGLLGPYIR" was obtained. Here a S/N ratio of 27 was found which is above the acceptance threshold of 5 (Fig. 6). The accuracy and precision of the calculated concentrations of standards 1 to 7 were also in agreement with guidelines (table 2). Within-run and between-run precision and accuracy were validated in three days using four QC levels in fivefold. The overall precision and accuracy for LLOQ, QC low, med and high were found to be within acceptance criteria of <20% and 15%, respectively(table 3). Combined standard uncertainty, which consists of the bias and CV, was calculated through the Nordtest approach and can be used to derive uncertainty values of sample measurements between de validated calibration range 1 – 500 ng/mL (table 4). Matrix effect was investigated by spiking known amounts of FVIII to citrate plasma from 3 different volunteers. No matrix

effect was observed; all calculated values corresponded well with spiked values (table 5).

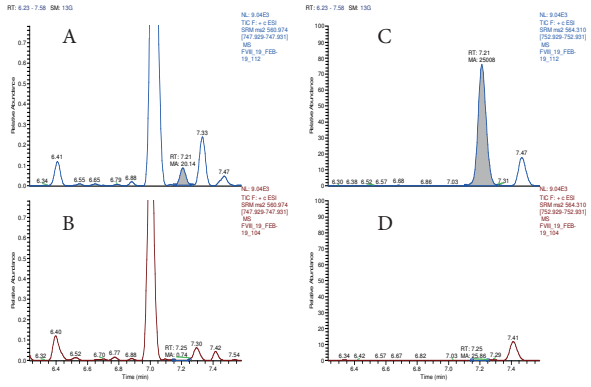

Figure 6. Left side, chromatogram of standard 1 ng/mL (A) and chromatogram of blank depleted FVIII plasma (B) both measuring GELNEHLGLLGPYIR SRM transition 561 → 747.93. Right side, chromatogram of internal standard (C) and chromatogram of blank (D) measuring GELNEHLGLLGPYI(R*) SRM transition 564 → 752.93.

Table 2. Statistics of the back calculated concentrations of the standard curve analyzed during three days.

	Nominal concentration (ng/ml)						
	500	200	80	40	16	4	1
Mean	506.97	194.42	74.81	39.16	15.76	4.34	1.00
Standard deviation	9.45	3.43	0.56	1.14	0.44	0.26	0.04
Accuracy (%)a	1.39	-2.8	-6.5	-2.1	-1.5	8.5	0.1
Imprecision (%)b	1.9	1.8	0.8	2.9	2.8	6.1	4.2

a Accuracy: Determined as (measured conc. – nominal conc.)/nominal conc. ×100%
b Imprecision: Expressed as co-efficient of variation (CV)

Table 3. Accuracy and precision validation data for QC's at LLOQ, Low, Medium and High levels. 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	14.5	13.5	19.8	3.3
Low	7.4	5.3	9.1	-4.3
Med	3.2	5.7	6.5	-4.5
High	2.2	4.6	5.1	-4.7

Table 4. QC data of within-run and between run accuracy and precision for the calculation of the combined standard uncertainty through the Nordtest approach.

Nominal value [ng/mL]	Day	#1	#2	#3	#4	#5	CV [%]	Bias [%}
LLOQ	1	0.995	0.680	1.075	1.097	0.900		
1	2	1.351	1.148	1.106	1.179	1.275	18.46	3.33
	3	1.187	0.839	1.045	0.824	0.800		
QC Low	1	4.807	4.975	5.136	5.217	5.508		
5	2	4.70	4.85	4.36	4.80	4.27	8.62	-4.30
	3	3.88	4.44	4.84	5.06	4.95		
QC Med	1	154.43	142.24	145.94	151.86	143.06		
150	2	125.20	136.42	132.64	136.18	137.59	5.79	-4.48
	3	147.09	152.21	146.18	152.05	146.01		
QC High	1	289.01	277.16	275.32	271.04	276.14		
300	2	281.57	280.50	265.73	287.29	278.00	4.51	-4.66
	3	303.80	304.81	302.93	301.59	295.20		
RMSCV		10.83 %			RMSCV=√(Σ(CVi)2/n)			
RMSbias		4.23%			RMSBias=√(Σ(biasi)2/n)			
Combined Standard Uncertainty		11.62%			Combined Standard Uncertainty=√(RMS2CV+RMS-2bias)			

Table 5. Matrix effect, each sample spiked at 3 different concentration levels

	Nominal concentration (ng/ml)			
Sample	Spike [ng/mL]	Spike [ng/mL]	Spike [ng/mL]	Rec [%]
# 1	290	564.8	570.5	101.0
	145	419.8	431.7	102.8
	72.5	347.3	351.2	101.1
	0	274.8	274.8	100.0
# 2	290	543.2	521.2	96.0
	145	398.2	395.7	99.4
	72.5	325.7	325.3	99.9
	0	253.2	253.2	100.0
# 3	290	578.8	551.1	95.2
	145	433.8	421.9	97.2
	72.5	361.3	346.7	95.9
	0	288.8	288.8	100.0

Conclusion and discussion

Here we describe for the first time the use of LC-MS/MS for the quantification of FVIII in citrated plasma. Critical method parameters were optimized and the resulting method was subjected to validation according to EMA guidelines. All parameters were found to be well within predefined acceptance criteria. The method, which utilizes camelid anti FVIII nanobodies for sample purification and LC-MS/MS for measurement is highly selective. The removal of interfering plasma proteins lowered the detection threshold significantly, resulting in similar sensitivity as the one-stage activity assay. The lower limit of quantification of FVIII was 1 ng/mL which corresponds to 3.6 fmol/mL, which to the best of our knowledge has never been reported before in the analysis of biopharmaceuticals with LC-MS/MS. This was mainly achieved by using an easy and robust sample processing method which consisted of an efficient immunoaffinity purification in combination with a highly sensitive mass spectrometer. This method was developed for an ongoing study to investigate the pharmacokinetics of FVIII and its added value to existing activity-based diagnosing and monitoring. Furthermore, LC-MS/MS method enables 'telemonitoring' of patients by means sampling at home using dried blood spot sampling for instance. Finally, multiplexing capabilities of LC-MS/MS would allow for other coagulation factors to be included in the same assay thus providing a way to quantitate multiple coagulation proteins in patient plasma in one analysis.

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General discussion and future perspectives

General Discussion

The quantification of therapeutic and endogenous proteins in biological matrices is important for diagnosis and treatment of patients with various diseases. Therapeutic protein such as recombinant proteins or monoclonal antibodies can induce immunogenicity in patients. This leads to the production of anti-drug antibodies which in turn accelerate drug clearance thus rendering the treatment ineffective. Quantification methods based on ligand binding assay are widely used to monitor therapeutic and endogenous protein concentration but have some important drawbacks such as cross-reactivity, narrow linear dynamic range, long assay development time, lack of multiplexing abilities and higher imprecision. Arguably, the most serious limitation is the inability of these assays to measure the component of interest directly. This leaves room for possible lack of specificity due to cross-reactivity. Cross-reactivity occurs when the binding or detecting antibody used in the assay has competing affinity toward different plasma proteins that contain similar structural regions as the target protein. Therefore, this thesis focused on the development of quantification methods for proteins based on liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS systems based on triple quadrupole, measures the signal intensity of signature peptides originating from the target protein. If the signature peptide is chosen correctly, then its intensity is directly correlated with the target protein concentration. However, wrong signature peptides could invalidate the test results. Indeed, if the chosen peptide is susceptible to degradation by means of deamidation or oxidation then the signal intensity of the measured mass transition could be lowered. Another important consideration is the various protein isoforms that exist of the target protein. Protein can be expressed with variable degrees of phosphorylation and glycosylation. Therefore, peptides with amino acid sites that are sensitive to post translation modification should be avoided.

The incorporation of an internal standard is also a powerful option that is frequently employed in LC-MS/MS methods. Internal standards can correct for variations in the sample pre-treatment and analysis. These variation can occur during sample purification, where for example the target protein can be lost during the washing steps. Or during digestion where the target protein is not completely digested. Also, LC-MS/MS instrumental variations, such as injection and ionisation suppression, can be corrected for with an internal standard. Furthermore, the overall variation in the method, which is a good quality control parameter, can also be monitor by comparing the signal responses of the internal standard throughout the run. Another advantage of LC-MS/MS system is that they can incorporate the same immunoaffinity sample purification as the ones used in ligand binding assay. This could allow

for easy method transfer to the more selective and precise LC-MS/MS system. Alternatively, LC-MS/MS methods can also incorporate a generic sample pre-treatment based on physical and or chemical characteristics of the protein such as size, charge, polarity and hydrophobicity. This is a powerful option that does not exist in ligand binding assay and therefore the method development time can be significantly shortened. In this thesis, several challenges in bioanalysis of proteins (such as appropriate sample purification, chromatographic separation and detection) with LC-MS/MS are highlighted. Furthermore, various examples of quantitative proteomics with LC-MS/MS were presented providing a way to overcome these challenges. Different sample purification techniques based on selective purification or protein fractionation were introduced. Bottom-up quantitative proteomics, through signature peptide measurement was used as basis for method development. Importantly, method optimization steps were provided in each chapter to illustrate how an experiment can be designed to determine the optimum conditions for binding, washing, denaturation and digestion thus allowing for successful method development.

Therapeutic antibody quantification

In chapter 2, a tutorial, including a review of the literature, based on the widely used bottom-up quantitative proteomics that can be used as guidance for method development is presented. This principle was chosen because it solves problems with chromatographic separation and sensitivity issues encountered in top-down and middle-down quantification. Furthermore, this principle only requires an LC coupled to a tandem mass spectrometer which can readily be found in PK and therapeutic drug monitoring laboratories.

The development of a method to quantify active infliximab (IFX) in human plasma was also introduced in this chapter. The therapeutic monoclonal antibody IFX is a widely used drug for the treatment of various inflammatory autoimmune diseases. However, due to its chimeric nature, consisting of mouse variable light and heavy chain framework region it can induce immunogenicity in patients which render the treatment ineffective. Therefore, personalized medicine is essential for this drug and this can only be achieved through therapeutic drug monitoring of the active IFX fraction in plasma. A target based sample purification method was used. Results correlated well (r^2=0.95) with results obtained with an enzyme linked immunosorbent assay (ELISA). However, LC-MS/MS results were a factor 1.5× higher compared to ELISA. After a literature search, we found that the same factor was also observed when ELISA assays from different manufacturers were compared [1]. This indicated that the differences found between ELISA and LC-MS/MS could be explained by the assay design. Since the reference ELISA used for comparison requires two free IFX epitopes for measurement

while for the sample purification in the LC-MS/MS method only one free IFX epitope was required, different fractions may be measured. Presumably, ELISA misses the IFX fraction with one TNF-α attached and thus it underestimates the concentration of active IFX. IFX with one free epitope can still bind to a second TNF-α and trigger cell lysis trough complement-dependent cytotoxicity and cell necrosis and apoptosis trough antibody-dependent cell-mediated cytotoxicity. An alternative LC-MS/MS methods for the quantification of IFX in rat serum using protein A sample purification has already been presented [2]. Protein A purifies immunoglobulin fraction G (IgG) from serum on the basis of its Fc region and does not distinguish between active or inactive IFX, measuring instead the total antibody fraction. Since reference therapeutic windows were already established based on active IFX fractions a targeted assay based on immunoaffinity purification was selected [3].

Patients who develop anti-drug antibodies (ADA) and become insensitive to IFX therapy may switch to the fully human therapeutic monoclonal antibody adalimumab (ADM). Therefore, in the following sub chapter a multiplexed method was developed to simultaneously quantify free ADM and IFX in human plasma [4]. Even though patients are infused with either IFX or ADM, the possibility to perform a batch run consisting of both IFX and ADM treated patients, significantly reduced analysis time. Here, the existing IFX method was optimized by increasing the 96-well plate binding capacity. Furthermore, the elution and denaturation conditions were simplified by eluting with formic acid thus eliminating the drying step and replacing the DTT reduction step with heat denaturation. This has led to the simplification of the method resulting in ease of use and lower chance for errors. Moreover, chromatographic run time was reduced to 5 minutes allowing the analysis of an entire 96 well plate in 8 hours. This might seem long compared to ELISA, however it is important to note that this time consists entirely of instrument time and can be performed overnight. Also, since these patients are on maintenance therapy, with drug administration every 2 - 8 weeks, a fast result is not mandatory.

The quantification of T-cell binding polyclonal rabbit anti thymocyte globulin (ATG) posed a different challenge. Here, the variable domains of the rabbit polyclonal immunoglobulins were not identical to each other. So, a signature peptide from this location was not feasible since it can only represent one ATG that targets one specific receptor. A solution was found by choosing a signature peptide from the constant chain to represent all rabbit IgG. Furthermore, due to differences in the constant chain frame work regions between rabbit and human IgG's multiple signature peptide candidates were available. However, only the T-cell binding (active) ATG fraction has been associated with the rate of immune reconstitution and graft failure, so a selective sample purification with Jurkat cells was mandatory.

These above mentioned methods all rely on a 'bait and prey' principle to capture the analyte of

interest. However, these antigens or anti-idiotypic antibodies are not commercially available for some therapeutic monoclonal antibodies.
Therefore, in the following chapter an alternative analytical procedure was developed to quantify total dinutuximab (DNX) in neuroblastoma patients using a generic method based on ammonium sulfate (AS) sample purification [5]. Here, large proteins were fractionated from small proteins with AS by changing their solubility in solution. Increasing concentration of AS causes heavy proteins to precipitate out of solution while leaving small proteins in solution. After centrifugation, albumin which account for approximately 60% of plasma proteins, was removed in the supernatant layer, leaving the IgG's behind in the pellet. The IgG fraction retained its tertiary conformation and unlike the widely used methanol precipitation [6-14] or isopropanol precipitation [15], the pellet was easily re-dissolved in buffer solution for efficient reduction and alkylation. The signature peptide used in DNX quantification originates from the heavy variable chain which was hard to digest completely. Here, sodium dodecyl sulfate (SDS) completely unfolded the protein which allowed trypsin easy access to the cleavage sites resulting in efficient digestion. This was a cheap alternative compared to commercially available MS compatible surfactant Rapigest™ [16-19]. However, the removal of SDS was necessary prior to trypsin digestion and MS measurement since SDS can also unfold trypsin and can cause sever MS ionization suppression.

Endogenous Protein Quantification

The quantification of endogenous proteins has additional challenges. Usually, the blank matrix (plasma) already contains the protein of interest, so preparing standard solutions from volunteers derived plasma is not feasible. Furthermore, the preparation of standard solutions is usually done with recombinant proteins which are not identical to the endogenous protein. These differences can influence method accuracy and need to be carefully evaluated.
For the quantification of neutralizing anti-drug antibodies in patients treated with IFX, it was possible to obtain a blank plasma from volunteers. However, it was impossible to obtain the exact same neutralizing antibodies that each patient developed against IFX. This method, which was based on the simultaneous quantification of ADM and IFX, was developed to provide insight in the neutralizing capacity of the anti-drug antibodies expressed as amount of IFX neutralized. This is arguably a more meaningful measure than the arbitrary unit per milliliter (AU/mL) measure used to express the concentration of anti-drug antibodies determined with radio-immunoassay. Indeed, knowing the capacity to neutralize a certain drug could open doors to new treatment strategies. Quantification of the neutralizing capacity of anti ADM antibodies through this strategy was not feasible since patients that are switched

to ADM therapy were primarily IFX treated patients that have developed anti-drug antibodies to IFX and therefore SIL IFX was not a suitable internal standard. Nevertheless, this method provided a way to simultaneously measure three components in one run, namely ADM, IFX and the neutralizing capacity of anti IFX antibodies, which was made possible though the multiplexing abilities of the LC-MS/MS.

The development of a method to quantify the coagulation FVIII concentration in plasma was setup for an ongoing study to determine whether concentration of FVIII is a better predictor for bleeding phenotypes compared to the existing one stage clotting assay (OSA), which is based on FVIII activity instead of concentration.

For the quantification of coagulation factor VIII (FVIII) in plasma, a commercial FVIII depleted pooled human plasma was found which was used for the preparation of calibration standards. However, after validation it was still uncertain if the reported value was accurate. This is due to multiple reasons. Firstly, the obtained standard was a recombinant protein and supplied as lyophilized powder. During the purification processing, the protein structure could have been damaged and would thus influence its ability to convert into active FVIII (FVIIIa). This in turn would result in signal loss due to inefficient immunoaffinity purification. Secondly, patients' FVIII was conjugated with Von Willebrand factor, while this was not the case for the standard samples. These differences could also influence the way the standard is converted to FVIIIa and thus its purification recovery.

There are ways to correct for these biases, namely by harmonizing the standard with a certified reference material (CRM) or by inter laboratory comparisons test. Unfortunately, for FVIII these options were not available yet since both CRM and inter laboratory comparisons are based on FVIII activity only. Even though a bias might exist, the method was precise and was well correlated with activity. For therapeutic drug monitoring, this would not be an issue if a therapeutic window can be established with the same method. In contrast to OSA, LC-MS/MS would allow for dried bloodspot analysis since FVIIIa will be present on the filter paper. Dried bloodspot offers the comfort and ease of home sampling and due to fewer hospital visits, significantly reduces costs for both patient and hospital [20]. Finally, multiplexing capabilities of LC-MS/MS would allow for other coagulation factors to be included in the same assay, thus providing a way to quantitate multiple coagulation proteins in patients' plasma in one analysis.

Future perspectives

Bottom-up quantitative proteomics which relies on enzymes to digest the protein into peptides is currently the most widely used approach. However, the development of an LC-MS/MS analytical procedure for quantification of a therapeutic or endogenous proteins trough this strategy is currently very time consuming due to the various steps that need to be optimized. The challenge starts with finding a suitable signature peptide that is unique for the target protein and that delivers a high signal to noise ratio. This requires screening of multiple peptide candidates and optimizing their LC separation and MS signal. For human or humanized therapeutic monoclonal antibody the choices for signature peptide are limited and there are cases where none could be found [21]. Furthermore, optimal digestion conditions can be different from one protein to another.

Middle-down quantitative proteomics which measures only a portion of the protein might in the future provide a way to overcome these challenges. After disulfide bond reduction, the light chain of a therapeutic monoclonal antibody could be quantified using high resolution mass spectrometer such as an orbitrap. The light chain which is usually devoid of glycans can be deconvoluted to its monoisotopic mass thus providing an accurate way to monitor its signal intensity. However, due to the various precursors that are formed during ionization, orbitrap sensitivity needs to increase to allow for the required detection limits. Also increased sampling rate would be needed to allow for short run times. Time of flight mass spectrometers have a superior scan rate compared to the orbitrap, but unfortunately their resolution is not sufficient to allow for monoisotopic mass deconvolution of the light chain. Fragmentation might provide a solution, by measuring a fragment of the light chain instead of the entire light chain, then monoisotopic mass deconvolution with lower resolution would be possible. Importantly, fragment measurement would also provide an additional degree of separation. However, the commonly used collision induced dissociation (CID) is not applicable here since only the nonspecific outer peptides of the light chain are released. Electron-induced fragmentation (ETD) provides a more efficient fragmentation, however this is mostly reserved to high end mass spectrometers. With increased competition there will be a need to innovate and these techniques will start to be implemented in lower end models.

Sample purification is another tedious part in method development. Strategies based on immunoaffinity interaction are very effective, delivering high recovery and selectivity. However, the required ligand and anti-iodiotypic antibodies are not always available, are expensive and the sample purification is time consuming to optimize and to perform. Multidimensional liquid chromatography may provide an alternative. These systems can be used to purify the target endogenous proteins from the biological matrix based on their physiological properties such as size, charge, hydrophobicity and polarity and can be used as standalone devices or can

be integrated with a mass spectrometer for online measurement. Since these systems are not regularly used, the software development is lagging. Most system providers offering heart cut injections only, which eliminates the possibility for simultaneous measurement of multiple target analytes. Another application for these systems is found in whole sample digest. Here, the entire sample is digested and peptides are separated by means of multidimensional liquid chromatography. Peptides differ more from each other in terms of polarity and charge than proteins do which would allow them to be separated more easily. Indeed, for therapeutic monoclonal antibodies for example this is not as straight forward since on average multiple IgGs might contain similar size, charge, hydrophobicity or polarity, making intact purification and quantification very challenging. However, for peptides one amino acid difference would greatly influence its physical characteristics resulting in baseline separation.

Implication for clinical practice

With gradual improvement and affordability of mass spectrometers, software and multidimensional chromatographic systems, method development will increasingly become easier and faster to perform. This in turn would make quantitative proteomics with LC-MS/MS more accessible. For PK research and therapeutic drug monitoring this could be beneficial for the patient since quantitative proteomics with LC-MS/MS need to pass rigorous validation protocol with higher precision and accuracy compared to assays such as ELISA. For diagnosis purposes a higher precision measurement would allow for accurate disease classification. For example, severe hemophilia A patients have a FVIII activity < 5%, while moderate hemophilia A patients have an activity >5%. Due to the greater method impression of the activity based assay at this lower end, more patients would be wrongfully classified with the activity based assay compared to the more precise LC-MS/MS method. The multiplexing capability of LC-MS/MS allows for multicomponent analysis, this reduces patient's sampling requirement and offers faster turnaround times. Finally, LC-MS/MS offers the possibility for method standardisation. Indeed, LC-MS/MS assays are not dependant on antibodies, which can vary between various ligand binding assays in terms of avidity and specificity. Therefore, LC-MS/MS analytical methods can easily be standardised between different laboratories.

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Summary

Summary

Therapeutic monoclonal antibodies are widely used for the treatment of various diseases were conventional small molecule based drugs are not effective. For patients with inflammatory autoimmune disease for example, the overproduction of TNF-alpha protein causes painful inflammatory symptoms. The level of TNF-alpha can be reduced through treatment with therapeutic monoclonal antibodies such as infliximab or adalimumab. However, some patients develop anti-drug antibodies that target these therapeutic 'exogenous' proteins thus reducing the drug concentration in plasma. This in turn renders the treatment ineffective. In this case quantitative proteomics is used for therapeutic drug monitoring (TDM) of drug concentration in plasma. This can provide the clinician useful insight to what is happening in the patient's body and can be used to tailor the patient's care.

Quantitative proteomics can also be used for endogenous proteins. Endogenous proteins are synthesized by the DNA within the cell nucleus. Endogenous proteins are the building blocks for all cells that make up the organism, they play a role in food digestion, they offer protection from pathogens and they heal the cells when damaged. The ability to determine whether these proteins are present in the required concentration in plasma can help the clinician to diagnose a disease but also to personalize the treatment plan. Patients with hemophilia A for example, have a mutation in the X chromosome in locus q28. This portion of the X chromosome codes for the synthesis of the 2351 amino acid long FVIII protein. However, if FVIII is not synthesized correctly, this happens when one amino acid is swapped for another, misshaped or misfolded FVIII protein might be formed which could compromise the binding efficiency to its stabilizer protein von Willebrand or to the other factors FIX and FX involved in coagulation, resulting in prolonged bleeding times. Different dosing schemes with recombinant FVIII are thus incorporated dependent on the level of FVIII deficiency.

This thesis provides a tutorial for quantitative proteomics with liquid chromatography–tandem mass spectrometry (LC-MS/MS), furthermore various examples of bioanalytical quantification of endogenous and therapeutic proteins in human plasma are presented. Novel sample purification strategies are introduced and optimized though experimental design. Some of these methods such as infliximab and adalimumab are already in use for routine TDM. Others, such as coagulation FVIII, neutralizing anti-drug antibodies, dinutuximab and active anti-thymocyte globulin (ATG) were developed for ongoing pharmacokinetics and pharmacodynamics studies.

In **chapter 2.1** a Six-step tutorial to quantify therapeutic monoclonal antibodies in biomatrices

was introduced. The promising pipeline of therapeutic monoclonal antibodies (mAbs) demands robust bioanalytical methods with swift development times for pharmacokinetic studies. Over the past decades ligand binding assays were the methods of choice for absolute quantification. However, the lengthy production of the required anti-idiotypic antibodies and ligands limits high-throughput method development. In recent years, high-resolution liquid chromatography tandem mass-spectrometry (LC-MS) systems have enabled absolute quantification of therapeutic mAbs with short method development times. These systems have additional benefits, such as a large linear dynamic range, a high specificity and the option of multiplexing. In this chapter, we briefly discuss the current strategies for the quantification of therapeutic mAbs in biological matrices using LC-MS analysis based on top-down and middle-down quantitative proteomics. Then, the widely used bottom-up method was presented in a six-step workflow, which can be used as guidance for quantitative LC-MS/MS method development of mAbs. Finally, strengths and weaknesses of the bottom-up method, which currently provides the most benefits, are discussed in detail.

In **chapter 2.2** an analytical procedure for the quantification of active infliximab in human serum with liquid chromatography-tandem mass spectrometry to facilitate therapeutic drug monitoring as part of the personalized medication treatment plan was developed. The therapeutic monoclonal antibody Infliximab (IFX) is a widely used drug for the treatment of several inflammatory autoimmune diseases. However, approximately 10% of patients develop anti-infliximab antibodies (ATIs) rendering the treatment ineffective. Early detection of underexposure to unbound IFX would result in a timely switch of therapy which could aid in the treatment of this disease. Streptavidin coated 96 well plates were used to capture biotinylated-tumor necrosis factor alpha (b-TNF-α), which in turn was used to selectively extract the active form of IFX in human serum. After elution, IFX was digested using trypsin and one signature peptide was selected for subsequent analysis on LC-MS/MS. The internal standard used was a stable isotopic labeled IFX bio-similar. The assay was successfully validated according to European Medicines Agency (EMA) guidelines and was found to be linear in a range of 0.5 – 20 µg/mL (r2=0.994). Lower limit of quantification for the assay (<20% CV) was 0.5 µg/mL, requiring only 2 µL of sample. Cross-validation against enzyme-linked immunosorbent assay (ELISA) resulted in a high correlation between methods (r2=0.95 with a ρc=0.83) and the accuracy was in line with previously published results. In conclusion, a sensitive, robust and cost-effective method was developed for the bio-analysis of IFX with LC-MS/MS by means of a target-based pre-analytical sample purification. Moreover, low volume and costs of consumables per sample promote its feasibility in (pre)clinical studies and in therapeutic drug monitoring.

In **chapter 2.3** the multiplexing capacity of the mass spectrometer for the simultaneous quantification of free adalimumab and infliximab in human plasma was utilized. Therapeutic drug monitoring of tumor necrosis factor alpha (TNF-α) inhibitors such as adalimumab (ADM) and infliximab (IFX) is considered of added value for patients with systemic inflammatory diseases. In contrast to enzyme-linked immune sorbent assay (ELISA) methods, liquid LC-MS/MS methods allow simultaneous quantification of multiple target antibodies in one run and thus providing a higher sample throughput. A fast sample work-up strategy for the absolute and simultaneous quantification of ADM and IFX therapeutic monoclonal antibodies in human plasma samples using a target specific sample purification in combination with LC-MS/MS was described. The sample purification was based on the selective capture of ADM and IFX in human plasma or serum using biotinylated TNF-α (b-TNF-α) which was coated on a streptavidin 96-well plate. After elution, analytes were heat denatured and trypsin digested in order to obtain signature peptides for quantification. Stable isotopically labeled ADM and IFX were introduced as internal standard prior to sample purification. The linear dynamic rage for both analytes were 1 - 32 µg/mL with an excellent mean coefficient of determination, R2 = 0.9994 for ADM and 0.9996 for IFX. Within-run and between-run precision and accuracy were within acceptance criteria. Cross-validation against ELISA method showed a high between-method correlation R2=0.962 for ADM and R2=0.982 for IFX. This method provides an easy, efficient and cost effective workflow for therapeutic drug monitoring patients treated with ADM or IFX.

In **chapter 2.4** an analytical method was developed to quantify T-cell binding polyclonal rabbit anti-thymocyte globulin (ATG) in human plasma. The addition of rabbit anti-human thymocyte globulin to the conditioning regimen prior to allogeneic haematopoietic cell transplantation (HCT) has significantly reduced the risk of graft versus host disease (GvHD) and graft failure. However, ATG has a small therapeutic window. Overexposure of ATG post-HCT hampers T-cell immune reconstitution and has been associated with increased relapse rates and viral reactivations, whereas underexposure has been associated with an increased incidence of GvHD, both of which lead to increased mortality. ATG dosing is ideally based on absolute lymphocyte count (ALC) and patients weight, however this is insufficient to prevent under- and overdosing in some patients. Therapeutic drug monitoring (TDM) of T-cell binding (active) ATG plasma levels provides a means to optimize dosing for patients at high risk for graft failure to ensure timely T-cell immune reconstitution and subsequently increase survival chances post-HCT. This manuscript describes the first LC-MS/MS method to quantify the pharmacologically active fraction of polyclonal ATG in plasma. This was achieved through immunoaffinity purification of active ATG from plasma with Jurkat T-cells. After the binding and washing, samples were eluted, denatured and trypsin digested.

Signature peptides originating from the IgG constant chain were measured with LC-MS/MS. Critical method parameters such as binding time, ratio of Jurkat T-cells to ATG, and digestion time were optimized. Matrix effect, freeze/ thaw stability, intra- and inter-assay accuracy and precision were all within EMA guidelines. The method covered the therapeutic range of ATG and was validated at an LLOQ of 1 AU/mL with an overall CV and bias of 11.8% and -2.5% respectively. In conclusion, we developed a LC-MS/MS-based method to quantify active polyclonal rabbit ATG in human plasma. We suggest that this novel assay can be used to monitor and optimize dosing of ATG in clinical practice.

In **chapter 2.5** a method was developed to quantify the total therapeutic monoclonal antibody concentration in plasma. Dinutuximab (DNX) concentration in neuroblastoma patients was quantified using a generic sample purification method based on ammonium sulfate and corrected with an internal standard based on a SIL peptide instead of a SIL protein. Neuroblastoma is one of the most commonly found solid tumors in children. The monoclonal antibody DNX targets the sialic acid-containing glycosphingolipid GD2 expressed on almost all neuroblastoma tumor cells and induces cell lysis. However, the expression of GD2 is not limited to tumor cells only, but is also present on central nerve tissue and peripheral nerve cells explaining dinutuximab toxicity. The most common adverse reactions are pain and discomfort, which may lead to discontinuation of the treatment. Furthermore, there is little to no data available on exposure and effect relationships of DNX. We, therefore, developed an easy method in order to quantify DNX levels in human plasma. Ammonium sulfate (AS) was used to precipitate all immunoglobulins (IgG's) in human plasma. After centrifugation, supernatant containing albumin was decanted and the precipitated IgG fraction was re-dissolved in a buffer containing 0.5% sodium dodecyl sulfate (SDS). Samples were then reduced, alkylated and digested with trypsin. Finally, a signature peptide in complementarity-determining region 1 of DNX heavy chain was quantified on LC-MS/MS using a stable isotopically labeled peptide as internal standard. AS purification efficiently removed 97.5% of the albumin fraction in the supernatant layer. The validation performed on DNX showed that within-run and between- run coefficients of variation (CV's) for lower limit of quantification (LLOQ) were 5.5% and 1.4%, respectively. The overall CV's for quality control (QC) Low, QC Med and QC High levels were <5%. Linearity in the range 1 – 32 mg/L was excellent (r2>0.999). Selectivity, stability and matrix effect were in concordance with EMA guidelines. In conclusion, a method to quantify DNX in human plasma was successfully developed. In addition, the high and robust process efficiency enabled the utilization of a stable isotopically labelled (SIL) peptide instead of SIL DNX, which was commercially unavailable.

In **chapter 3.1** a proof of principle was provided for the quantification of neutralizing anti

infliximab antibodies and their neutralizing capacity using competitive displacement. The development of anti-drug antibodies (ADA) in patients treated with therapeutic proteins can result in treatment failure. The clinically most relevant fraction of these antibodies are the neutralizing anti-drug antibodies (NAb) that block the pharmacological function of the drug. Consequently, the detection of NAb in plasma is a better predictor of loss of therapeutic response than increased levels of total anti-drug antibodies (ADA) test. Traditional assays to detect ADA and NAb have limited specificity, sensitivity and linear dynamic range. This chapter demonstrates for the first time the potential of a LC-MS/MS method to measure the concentration of NAb against therapeutic proteins in plasma as exemplified with infliximab (IFX). A competitive screening assay in which the presence of NAb in patients plasma prevents the binding of stable isotopically labeled (SIL) mAb infliximab to TNF-α ligand fixed on a 96-well plate was designed. After washing, eluting and digesting, the signal intensity of SIL IFX-derived signature peptides was inversely and strongly correlated with NAb concentration in the sample: R2=0.999. Evaluation data showed that the assay has a high specificity (100%) and a high sensitivity (94%) to predict NAb presence. Cross-validation against total ADA measured by a reference laboratory using radio immunoassay assay (RIA) for ADA provided a good correlation (r2 = 0.79). In conclusion, for the first time a robust and fast screening method was developed on the basis of LC-MS/MS to determine the presence of NAb and its neutralizing capacity in plasma. The analyses of NAb can be combined with therapeutic mAb quantification. Furthermore, the quantification of the neutralizing capacity expressed as mAb mass equivalents opens the door to new personalized dosing strategies in patients with NAb.

In **chapter 3.2** a method to quantify the concentration of coagulation factor VIII in human plasma was developed. Patients with hemophilia A are currently diagnosed and monitored by measuring the activity of coagulation factor VIII (FVIII) in plasma mostly with the one-stage clotting assay (OSA). Although the OSA is routinely available in many clinical laboratories, it has in some circumstances relatively low sensitivity and specificity. Therefore, the FVIII activity as a biomarker does not always correlate with the bleeding phenotype. Hence, we have developed a LC-MS/MS method to quantify the concentration of coagulation FVIII in plasma which would allow us to investigate the relation between FVIII plasma concentration, FVIII activity and bleeding tendency in future studies. LC-MS/MS method was set up by firstly dissociation Von Willebrand factor (VWF) from coagulation factor VIII by triggering the coagulation cascade to occur thus generating active factor VIII (FVIIIa). FVIIIa was then selectively extracted by means of immunoaffinity interaction using anti-FVIII camelid nanobodies, after which FVIIIa was eluted, heat denatured and trypsin digested. Finally, a FVIII specific peptide was used as a surrogate for quantification by mass spectrometry. Critical method parameters such as antibody amount, incubation time, sample volume and

type of streptavidin 96 well plate were optimized. The method was validated according to European Medicines Agency (EMA) guidelines where an LLOQ of 1 ng/mL was obtained using 50 µL of citrate plasma sample. Within-run and between-run accuracy and precision for quality control (QC) samples, LLOQ (1 ng/mL), QC Low (5 ng/mL), QC Med (150 ng/mL), QC High (300 ng/mL) were within the threshold of 15% relative standard deviation (RSD) and Bias. The selective immunoaffinity method which was used in combination with a highly sensitive mass spectrometer allowed for an unpresented LLOQ of 1 ng/mL utilizing 50 µL plasma sample. This method will be used to investigate the added value of FVIII plasma concentration which may be used in conjunction with FVIII activity for patient diagnosis and dosage optimization.

Samenvatting

Samenvatting

Therapeutische monoklonale antilichamen worden veelvuldig gebruikt bij ziektes waar conventionele geneesmiddelen, op basis van kleine moleculen, niet effectief zijn. Voor patiënten met inflammatoire auto-immuunziekte bijvoorbeeld vindt er een overproductie plaats van het eiwit TNF-alpha. TNF-alpha veroorzaakt pijnlijk geïrriteerd weefsel en kan behandeld worden met verschillende therapeutische monoklonale antilichamen zoals infliximab en adalimumab. Deze antilichamen binden de TNF-alpha vast en zorgen ervoor dat deze sneller wordt opgeruimd uit het lichaam. Echter, sommige patiënten ontwikkelen zelf antilichamen tegen infliximab of adalimumab aangezien deze lichaamsvreemd zijn. Hierdoor daalt de geneesmiddelconcentratie tot het niveau dat deze niet langer werkzaam is. Door de concentratie van het geneesmiddel in de patiënt te monitoren kan de behandelaar inzicht krijgen in de blootstelling in het lichaam en kan daarmee een voor de patiënt gepersonaliseerd behandelingsplan worden opgesteld.

Het kwantificeren van endogene eiwitten is soms ook belangrijk. DNA in het lichaam codeert voor de aanmaak van eiwitten. Deze endogene eiwitten fungeren als bouwblokken voor het lichaam, ze bieden bescherming tegen infecties, ze zijn betrokken bij het verteren van voedsel, en ook bij genezing in het geval van weefselschade. Door het gehalte van deze endogene eiwitten te bepalen kan de arts genetische ziektes diagnosticeren en daarnaast ook een behandelingsplan voorschrijven. Bijvoorbeeld, bij patiënten met hemofilie A is er sprake van een mutatie in de X-chromosoom in locatie q28. Dit gebied van de X-chromosoom is verantwoordelijk voor de aanmaak van het 2351 aminozuur tellende eiwit FVIII. Echter, wanneer FVIII niet op de juiste manier wordt aangemaakt (dit gebeurt wanneer een bepaald aminozuur met een ander is omgewisseld), dan wordt een andere quaternaire structuur van het eiwit verkregen. Doordat de vouwing anders is dan het originele FVIII-eiwit kan de binding met andere stollingsfactoren zoals Von Willebrand, FIX of FX negatief worden beïnvloed, waardoor stolselvorming vertraagd wordt. Afhankelijk van de mate van FVIII-deficiëntie worden verschillende doseringen met recombinante FVIII voorgeschreven. In dit proefschrift zijn een handleiding en verschillende voorbeelden van bioanalyse van zowel endogene als therapeutische eiwitten met vloeistofchromatografie tandem-massaspectrometrie (LC-MS/MS) opgenomen. Verschillende opwerkingstechnieken worden hier geïntroduceerd met daarbij behorende experimentele optimalisaties. Deze opgezette analysemethodes worden momenteel gebruikt voor het monitoren en optimaliseren van geneesmiddelconcentraties en voor het bestuderen van de farmacokinetiek (PK) en farmacodynamiek (PD).

In **hoofdstuk 2.1** werd een zes-staps-handleiding geïntroduceerd voor het opzetten van een

LC-MS/MS-methode voor het kwantificeren van therapeutische monoklonale antilichamen in biologische matrices. Het bestuderen van de farmacokinetiek en farmacodynamiek van een nieuw geneesmiddel vergt een robuuste bioanalytische methode welke snel kan worden opgezet. Traditioneel gezien waren immunologische bepalingen de eerste en soms de enige keuze. Echter, vanwege hun afhankelijkheid van antilichamen of liganden duurde de methode-ontwikkeling lang. Systemen op basis van LC-MS hebben daar de laatste jaren verandering in gebracht. Deze systemen zijn in staat om een analytische methode, onafhankelijk van antilichamen, snel op te zetten en te valideren. Daarnaast hebben ze andere voordelen zoals een breder lineair dynamisch bereik, hoge specificiteit en de mogelijkheid om verschillende componenten tegelijkertijd te meten, ook wel multiplexing genoemd. In dit hoofdstuk werden de verschillende strategieën uitgelegd voor het kwantificeren van therapeutische monoklonale antilichamen in biologische matrices. 'Top-down', 'middle-down' en 'bottom-up' eiwitkwantificatie werden uitvoerig besproken en tot slot werd de meest gebruikte techniek op basis van bottom-up eiwitkwantificatie uitbreid beschreven.

In **hoofdstuk 2.2** werd een analytische procedure opgezet voor het kwantificeren van actieve infliximab (IFX) in humaan serum met LC-MS/MS. Dit werd gedaan om de geneesmiddelconcentratie in patiënten te kunnen monitoren als onderdeel van hun behandelingsplan. IFX is een therapeutisch monoklonaal antilichaam, dat gebruikt wordt voor de behandeling van ontstekingsziektes. Echter, ongeveer 10% van de behandelde patiënten ontwikkelt anti-IFX-antilichamen die de werking van het geneesmiddel tegengaan. Het vroegtijdig opsporen van sub-therapeutische concentraties van IFX kan ervoor zorgen dat patiënten beter behandeld kunnen worden. Streptavidin 96-well platen werden gebruikt om de gebiotinyleerde tumornecrosefactor alpha (TNF-α) te kunnen opvangen. Deze TNF-α werd vervolgens gebruikt om actief IFX uit humaan serum selectief te binden. Na elutie werd IFX met trypsine gedigesteerd en een voor IFX unieke peptide werd met LC-MS/MS geanalyseerd. Stabiele isotoopgelabelde (SIL) IFX werd als interne standaard gebruikt ter correctie voor eventueel verlies tijdens de opwerking. De methode werd volgens richtlijnen van het Europees Geneesmiddelenagentschap (EMA) gevalideerd en had daarnaast een goede correlatie met de traditionele immunologische assay (r2=0.95). Voor deze analysemethode is een klein monstervolume (2μL) voldoende waardoor deze toepasbaar is voor zowel preklinische studies als voor het monitoren van geneesmiddelen in patiënten.

In **hoofdstuk 2.3** werd de mogelijkheid van de massaspectrometer om simultane metingen te kunnen verrichten benut. Er is een methode opgezet voor de gelijktijdige meting van zowel actief adalimumab (ADM) als actief infliximab (IFX) in humaan plasma. Monitoring van plasmaconcentraties van TNF-α remmers zoals ADM en IFX is belangrijk bij patiënten

met systemische ontstekingsziektes. Een LC-MS/MS-systeem kan, ten opzichte van immunologische bepalingen, in een opgewerkt monster simultaan meerdere therapeutische antilichamen kwantificeren. Hierdoor kunnen veel meer monsters op een dag geanalyseerd worden. De bestaande IFX-opwerkingsmethode werd aangepast om zowel IFX als ADM te kunnen opzuiveren. Foutgevoelige stappen zoals de organische elutie en de reductie van disulfide bindingen werden vervangen door eenvoudige alternatieven. Het lineaire bereik werd verbreed van 1 – 20 µg/L naar 1 – 32 µg/L. De methode voldeed aan alle validatie-eisen en werd wederom met de bestaande immunologische bepaling vergeleken, waar een hoge correlatie werd gevonden voor zowel ADM R2=0.962 als IFX R2=0.982. Deze analytische bepaling is makkelijk uit te voeren, heeft een hoge mate van precisie en kan gebruikt worden voor de simultane meting van ADM en IFX ten behoeve van geneesmiddelenonderzoek in patiënten.

In **hoofdstuk 2.4** werd een analytische methode ontwikkeld voor het kwantificeren van T-cell bindende polyklonale anti-thymocytenglobuline (actief ATG) van konijnen. De toevoeging van ATG aan het conditioneringsregime voorafgaand aan stamceltransplantatie heeft geleid tot een significante vermindering van de graft-versus-host-reactie (GvHD) en transplantaatafstoting. ATG heeft echter een smal therapeutisch venster. Een te hoge blootstelling aan ATG voor stamceltransplantatie verhindert de immuunreconstitutie van T-cellen en is ook gelinkt aan een verhoogde kans op terugval en virale reactivatie. Bij een te lage blootstelling aan ATG is er een verhoogde kans op GvHD. Beide gevallen zijn geassocieerd met een toenemende sterfte. De ATG-dosering wordt idealiter gebaseerd op de absolute lymfocytenaantallen (ALS) en het gewicht van de patiënt. Bij sommige patiënten is dit echter onvoldoende om de juiste dosering te garanderen. Het kwantificeren van actief ATG in plasma biedt de mogelijkheid om de dosis voor patiënten met een verhoogd risico op transplantatiefalen te optimaliseren. Dit hoofdstuk beschrijft de eerste LC-MS/MS-methode om actief polyklonaal konijn-ATG in plasma te meten. Unieke peptides die representatief zijn voor konijn-IgG werden gebruikt voor kwantificatie. Een selectieve monsteropwerking met Jurkat T-cellen werd gebruikt om alleen de actieve ATG te kunnen opvangen. Na elutie van actief ATG werden deze met trypsine gedigesteerd en daaruit werden unieke peptides afkomstig van de constante keten van konijn-IgG gemeten met LC-MS/MS. Na optimalisatie werd de methode gevalideerd volgens Europese richtlijnen. Deze methode kan gebruikt worden om de ATG-concentratie in plasma van klinische patiënten te monitoren om de dosering te individualiseren.

In **hoofdstuk 2.5** werd een universele analytische procedure ontwikkeld voor het kwantificeren van het totale gehalte aan monoklonaal therapeutisch antilichaam in plasma. Dinutuximab-

concentraties (DNX) in neuroblastoma-patiënten werden gekwantificeerd met een generieke methode op basis van ammoniumsulfaatprecipitatie. Daarnaast werd een SIL-peptide, die makkelijker verkrijgbaar is dan een SIL-eiwit, gebuikt als interne standaard. Neuroblastoom is een van de meest voorkomende vaste tumorsoorten bij kinderen. Het monoklonaal antilichaam DNX richt zich op het binden van GD2-membraanlipide. GD2 bevindt zich in hoge mate op dit type tumoren en wanneer DNX aan GD2 gebonden is, kan celdoding op gang komen. Echter, GD2 bevindt zich ook op andere weefsels zoals zenuwcellen, waardoor patiënten heftige pijn kunnen ondervinden tijdens de behandeling. Aangezien de relatie tussen blootstelling en effect weinig onderzocht is, werd er een analytische procedure op basis van LC-MS/MS opgezet om hier meer inzicht in te krijgen. Ammoniumsulfaat werd gebruikt om alle immunoglobulines (IgG) inclusief DNX in humaan plasma neer te slaan. Na het centrifugeren van het opgewerkte monstermateriaal werd een IgG-pellet verkregen met daarboven albumine in oplossing. Nadat de bovenste laag was afgegoten, kon de IgG-fractie worden opgelost in een bufferoplossing. Daarna werd DNX gereduceerd, gealkyleerd en tot slot gedigesteerd met het enzym trypsine. Een voor DNX unieke peptide welke na digestie werd vrijgemaakt werd gemeten op de LC-MS/MS. De analytische bepalingsmethode werd gevalideerd volgens Europese richtlijnen en alle validatieparameters werden goed bevonden. Tot slot kan deze methode gebruikt worden om totaal DNX of andere therapeutische antilichamen in humaan plasma te kwantificeren, gebruikmakend van materialen die gemakkelijk te verkrijgen zijn.

In **hoofdstuk 3.1** werden gebruikmakend van een competitieve assay neutraliserende anti-IFX-antilichamen (NATI) en ook hun neutraliserende capaciteit bepaald. Patiënten die behandeld worden met therapeutische eiwitten kunnen tegen de medicatie antilichamen (anti-drug antibodies (ADA)) ontwikkelen waardoor die niet langer werkzaam zijn. Klinisch gezien zijn vooral de neutraliserende antilichamen (NAb) erg interessant, omdat deze in staat zijn de werking van het geneesmiddel te blokkeren. Hierdoor zou de NAb-concentratie in patiënten een betere voorspeller zijn voor werkzaamheidsverlies van een geneesmiddel dan de huidige totale ADA-concentratie. Huidige analytische methodes voor het kwantificeren van ADA en NAb hebben een lage specificiteit, gevoeligheid en een kort lineair dynamisch bereik. In dit hoofdstuk werd voor het eerst een analytische methode op basis van LC-MS/MS gedemonstreerd voor het kwantificeren van NAb tegen IFX (NATI). Wanneer NATI in patiëntplasma aanwezig is, zal deze aan een toegevoegde hoeveelheid SIL IFX binden. Afhankelijk van de hoeveelheid NATI, blijft een hoeveelheid SIL IFX over die opgezuiverd kan worden met TNF-α gebonden 96-well platen. Hierdoor is na de LC-MS/MS-meting een afname in signaal van SIL IFX-peptide te zien in verhouding tot de NATI-hoeveelheid in patiëntenplasma. De analytische procedure had een hoge mate van specificiteit (100%) en

sensitiviteit (90%) om NAb-aanwezigheid te voorspellen. Verder werd de methode vergeleken met een immunologische methode en hierbij is een goede correlatie gevonden (r2 = 0.79). De mogelijkheid om de neutraliserende capaciteit van NAb te kunnen bepalen kan in de toekomst bijdragen aan nieuwe gepersonaliseerde behandelingsstrategieën.

In **hoofdstuk 3.2** werd een methode ontwikkeld voor het kwantificeren van de stollingsfactor VIII (FVIII) in humaan plasma. Patiënten met hemofilie A worden momenteel gediagnostiseerd en gemonitord door de activiteit van FVIII in plasma te meten met een 'one-stage clotting assay' (OSA). Alhoewel de OSA routinematig in verschillende laboratoria wordt gebruikt, heeft deze in sommige gevallen relatief lage specificiteit en sensitiviteit waardoor de bepaalde FVIII-activiteit niet goed correleert met het bloedingsfenotype. Een analytische methode om de concentratie FVIII in plasma te kunnen bepalen met LC-MS/MS werd daarom opgezet. De LC-MS/MS-methode biedt de mogelijkheid om de relatie tussen FVIII-plasmaconcentratie, FVIII-activiteit en bloedingsneiging in toekomstige studies te kunnen onderzoeken. Eerst werd FVIII in actief FVIII (FVIIIa) omgezet door de Von Willebrand-factor los te maken en door FVIII, met het enzym trombine, in kleinere fracties te knippen. Daarna werd de vrijgekomen FVIIIa selectief opgevangen door immuuninteractie aan te gaan met een kamelenantilichaam. Na elutie en denaturatie middels hitte werd het eiwit met trypsine gedigesteerd en werd een specifieke peptide met LC-MS/MS gekwantificeerd. De analytische procedure voldeed aan de eisen voor validatie en had een nog niet eerder gerapporteerde gevoeligheid van 1ng/mL. Deze methode zal gebruikt worden om de toegevoegde waarde voor het diagnosticeren van patiënten en het optimaliseren van doseringen met FVIII-plasmaconcentratie te onderzoeken.

Dankwoord

Dankwoord

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M. El Amrani, R. Admiraal, Lobke Willaert, Lysette J.C. Ebskamp-van Raaij, Amelia M. Lacna, C. E. Hack, A.D.R. Huitema, S. Nierkens, E.M. van Maarseveen, Quantification of T-cell binding polyclonal rabbit anti-thymocyte globulin in human plasma with liquid chromatography–tandem mass spectrometry, The AAPS Journal (2020) 22:43

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labeled infliximab bio-similar as internal standard: A target-based, sensitive and cost-effective method, Journal of Chromatography A, 1454 (2016) 42-48.

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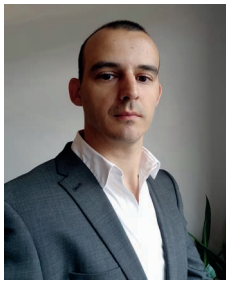
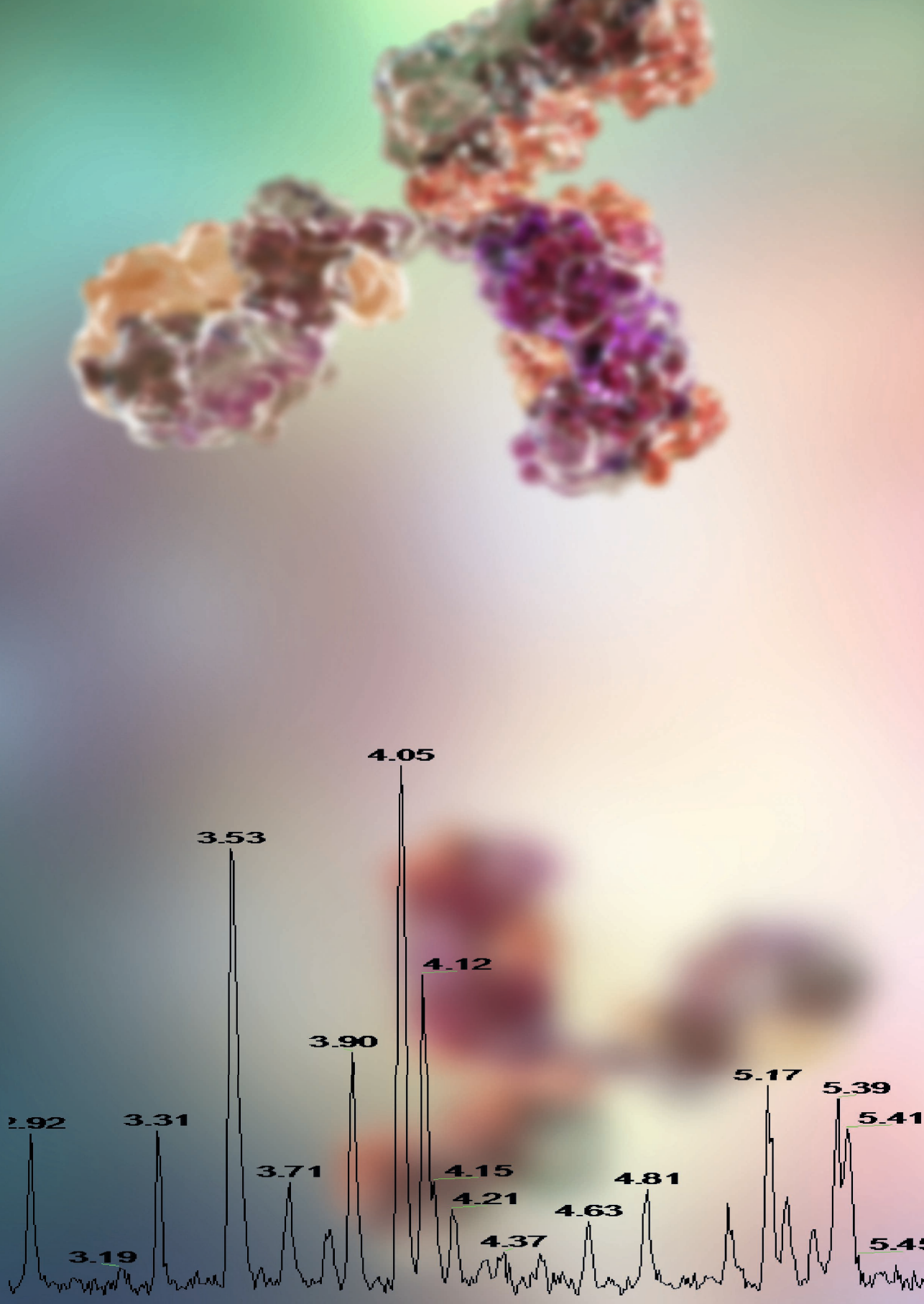
About the author



Mohsin El Amrani was born in Utrecht, the Netherlands on September 18, 1978. He obtained a high school degree (mavo) at the "Delta Collage" in Utrecht in 1995. In 1999 he completed a degree at "ROC Utrecht", obtaining an mlo diploma as a laboratory technician. In 2002 he obtained a Bachelor degree (hlo) at the institute of life sciences & chemistry (Hogeschool van Utrecht) majoring in analytical chemistry. In 2014 he obtained a Master's degree in analytical sciences at the "Vu" University in Amsterdam.

Mohsin started his professional career in 2003 as a laboratory technician and in 2005 became a senior analytical chemist at Analytical Research laboratories (ARL) in Napier, New Zealand. There, he became proficient in inorganic chemical analysis on ICP-AES and in feed quality analysis using near infrared spectrometry (NIR). In 2008 he took on a position as an analytical chemist at "Tergooiziekenhuizen" in Blaricum, The Netherlands, where he later became a research analyst. There, he supervised development and validation of bioanalytical methods for therapeutic drug monitoring (TDM). From 2011 to 2014 he was employed as a routine TDM analyst for the University Medical Center in Utrecht (UMCU) and within that same time frame he completed a master study in analytical chemistry at the Vu in Amsterdam. His master thesis project at the UMCU focused on the suitability of LC-MS/MS for the analysis of biopharmaceuticals in serum and subsequently went on to develop a LC-MS/MS method for the quantification of infliximab. After the completion of his master's degree, he was promoted to research analyst position and in February 2017, was enrolled in a PhD track. Mohsin lives together with his partner Salima in Den Haag and enjoys going to the park, zoo and swimming pool with his loving daughter Asya.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