High-sensitivity quantitation of a biopharmaceutical Nanobody® in plasma by single-cartridge multi-dimensional solid-phase extraction and UPLC-MS/MS

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Research Article

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Bioanalysis

High-sensitivity quantitation of a Nanobody® in plasma by single-cartridge multidimensional SPE and ultra-performance LC–MS/MS

Background: A major challenge in protein quantitation based on enzymatic digestion of complex biological samples and subsequent LC–MS/MS analysis of a signature peptide is dealing with the high complexity of the matrix after digestion, which can reduce sensitivity considerably. **Results:** Using single cartridge multidimensional SPE, sufficient selectivity was introduced to allow quantitation in 50 µl of plasma down to 10.0 ng/ml (~0.3 nM). An inhouse prepared ^{18}O-labeled signature peptide was used as the internal standard. The procedure was validated for human and rabbit plasma. **Conclusion:** The developed SPE procedure allowed the sensitive and selective LC–MS/MS quantitation of the Nanobody® without the use of antibodies. When appropriate precautions are taken, the ^{18}O-labeled peptide is a practical and economical alternative to custom synthesis.

Keywords: antibody-free • biopharmaceutical • enzymatic digestion • high sensitivity • LC–MS/MS • multidimensional SPE • Nanobody • oxygen exchange internal standard

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Nanobodies® are therapeutic proteins based on the smallest functional fragments of heavy-chain antibodies, which show great promise for several therapeutic areas such as oncology, inflammation and hematology. The Nanobody technology was originally developed following the discovery that camelidae (camels and llamas) possess fully functional antibodies that lack light chains. The heavy-chain antibodies in these animals contain a single variable domain and two constant domains. Importantly, the cloned and isolated variable domain is a stable polypeptide harboring the full antigen-binding capacity of the original heavy-chain antibody. These proteins form the basis of a new generation of therapeutic antibodies, termed Nanobodies [1].

Compared with monoclonal antibodies, which constitute an established biopharmaceutical platform, Nanobodies have several advantages. Owing to their reduced size (14 vs 150 kDa) and the absence of glycosylation, Nanobodies can be produced in expression systems such as in *Escherichia coli,* which is considerably more cost effective and straightforward than in cell cultures of more complex organisms. Furthermore, the same advantages apply to characterization of the finished product. Nanobodies consisting of two or more 14-kDa domains can be produced, which allows the combination of identical or different functional domains in one protein [2]. This technique can, for example, be used to increase avidity [3] by combining two identical Nanobodies, or to extend the half-life of the protein by combining it with an albumin-binding Nanobody [4].

In recent years, LC–MS/MS has received increasing interest as an alternative analytical platform for proteins in complex biological matrices [5–7]. This development is being driven by the better analytical performance of the technology, the possibility to obtain structural information, the potential for excluding antibody-based reagents, which may be difficult and costly to obtain, and the possibility to avoid interferences from protein–protein interactions such as antidrug antibodies and the circulating target.

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Key terms

Antidrug antibodies: Proteins produced by the immune system in response to the administered drug; when bound to the active site of the drug, they might induce loss of therapeutic effect.

Digestion: The process in which proteolytic enzymes cleave large proteins into small peptides, often by the enzyme trypsin owing to its highly predictable and reproducible cleavage behavior and the favorable average size and ionization behavior of the peptides it releases.

Signature peptide: A peptide that can only be released from the protein of interest and which is quantified by LC–MS as a measure for the intact protein.

Tween-20: An emulsifier that aids in the solvation of proteins and peptides, thus reducing adsorption to surfaces.

Because intact proteins are relatively poor candidates for mass spectrometric quantitation, owing to their size and molecular weight, high-sensitivity quantitative methods with LC–MS/MS require a proteolytic digestion step that cleaves the protein into smaller peptides, one of which, the signature peptide, is selected and subsequently used for quantitation.

Despite its potential, the use of LC–MS/MS for protein quantitation needs further development. Its main disadvantage compared with ligand-binding assays (LBAs) is its lower sensitivity, which is typically due to the limited selectivity of the technique for digests of protein-rich samples. Plasma and serum are very complex matrices that presumably contain several hundreds of thousands of proteins and protein isoforms [8]. Upon digestion these are all cleaved into multiple peptides, from which just one or a few have to be quantified. If no further clean-up is performed, the introduction of this multitude of peptides into the LC–MS/MS system will easily lead to interfering peaks in the chromatogram, ion suppression and an elevated baseline as a result of the MS response of coeluting peptides with very similar molecular properties, even with a relatively selective detection mode as SRM.

For high-sensitivity LC–MS/MS applications, several groups have reported immunoaffinity extractions to reduce the sample complexity either at the protein level [9,10] or after digestion to extract the signature peptide from the digested sample [11]. Although these approaches are capable of introducing the required selectivity, they do need the production and characterization of antibodies, which is a relatively time-consuming step. Furthermore, when applying immunoaffinity enrichment at the protein level, protein–protein interactions such as binding to circulating target and/or antidrug antibodies, can significantly interfere with binding to the immobilized antibody or target used for the enrichment. This interference also constitutes an important disadvantage of the LBA analytical platform.

As an alternative, more generic clean-up procedures can be applied, such as partial protein precipitation with which sensitivities down to 250 [12] and 50.0 ng/ml [13] were reached, or immune-based removal of abundant proteins, which resulted in a sensitivity of 4.0 µg/ml [14].

In our current work, we use proteolytic digestion of all proteins in the sample, which removes interferences from protein–protein interactions, as all proteins are cleaved into peptides. The selectivity required to achieve sufficient sensitivity from this highly complex mixture was reached by the subsequent application of three readily available orthogonal dimensions of separation: weak anion exchange and high-pH reversed phase interaction performed on a single mixed-mode SPE cartridge, plus high-resolution ultra-performance LC on a reversed-phase analytical column operated at low pH. Using this approach, a LLOQ of 10 ng/ml was obtained. Mixed mode [15] and 2D SPE [16,17] for clean-up of a digested sample have been described before, but with little to no discrimination between the different classes of peptides because of the conditions chosen for washing and eluting, leading to considerably higher LLOQ values (high ng/ml to µg/ml range). Furthermore, the single cartridge approach potentially reduces losses from repeated drying and redissolving required when multiple cartridges are used as well as time and cost reductions.

To correct for the analytical variability resulting from this procedure, an inhouse created stable-isotope labeled (SIL) internal standard was used, in which all six carboxyl-oxygen atoms of the signature peptide had been replaced by ^{18}O-atoms by acid-catalyzed oxygen exchange [18,19]. Application of this internal standardization approach required optimization of the conditions during sample pretreatment to prevent both chemical and enzymatic back-exchange, which is a well-documented problem of the technique [20].

Using this approach, a high-throughput, antibody-free and sensitive analytical method was obtained allowing the reliable quantitation of low ng/ml concentrations of a therapeutic protein in plasma and which was validated according to international guidelines for bioanalytical method validation [21]. The application of the method to support an early-phase preclinical trial in which the biopharmaceutical was administered intraocularly to rabbits is described, as well as the comparison of the LC–MS/MS results with those of a traditional LBA.

Experimental section

Chemicals & materials

The 259-amino acid, 28-kDa bivalent Nanobody was obtained from Ablynx (Ghent, Belgium) as a 26.6 mg/ml solution in phosphate-buffered saline (PBS). Ultrapure water was produced using an inhouse purification system (Sartorius, Göttingen, Germany). Acetonitrile and methanol were purchased from Biosolve (Valkenswaard, the Netherlands). Ammonia, ammonium acetate, sodium chloride, Tween-20, DMSO, ammonium bicarbonate, trypsin (non-N-Tosyl-L-phenylalanine chloromethyl ketone treated), hydrochloric acid (37%) and ^{18}O-labeled water were obtained from Sigma-Aldrich (St Louis, MO, USA). Heptafluorobutyric acid (HFBA) and formic acid were purchased from Merck (Darmstadt, Germany). Human and rabbit (New Zealand white) plasma with sodium citrate as the anticoagulant was obtained from Sera Laboratories (Haywards Heath, UK).

^{18}O-labeling of the internal standard

A 200-µl aliquot of a solution of the Nanobody (1 mg/ml in PBS) was pipetted into a 1.5-ml polypropylene cup and digested after the addition of 50 µl of a 1-mg/ml trypsin solution in 1 mM hydrochloric acid and 100 µl of 250 mM ammonium bicarbonate in water by placing it at 37°C and 900 rpm for 16 h.

The signature peptide was extracted from the digest by application of SPE on an Oasis® HLB, 30-mg 1-ml cartridge (Waters, Milford, MA, USA) using the following protocol. The cartridge was conditioned with 1 ml of methanol and 1 ml of 1% formic acid in water, after which the digest, diluted with 500 µl of 2% formic acid in water, was loaded. The cartridge was subsequently washed with 1 ml of 2% formic acid in water and 1 ml of 1% formic acid in a 10:90 (v/v) mixture of methanol and water. The signature peptide was eluted with 1 ml of 1% formic acid in a 50:50 (v/v) mixture of acetonitrile and water. The eluate was collected in a 2-ml polypropylene cup, and evaporated to dryness at 50°C under a gentle flow of nitrogen. The dried extract was reconstituted in 250 mg of ^{18}O-labeled water and, after adding 4 µl of HFBA, the cup was sealed and placed at 50°C for 47 h.

After the exchange was complete, the reaction was stopped by the addition of 1 ml of a solution of 100 mM ammonium bicarbonate, 10% acetonitrile and 0.02% Tween-20 in water. The solution was stored at -20°C. An internal standard working solution was prepared by 40-fold dilution of this solution with 100 mM ammonium bicarbonate in water.

Calibration & validation samples

A stock solution of 1 mg/ml of the Nanobody was made by diluting the reference solution with PBS. Eleven calibration standards were prepared in the range of 10.0 to 10,000 ng/ml by serial dilutions of this stock solution in blank human plasma. The validation samples were prepared at four concentrations (10.0, 30.0, 800 and 8000 ng/ml) from a separate 1-mg/ml stock solution. The stock solutions and prepared plasma samples were aliquoted and stored in polypropylene tubes at -80°C.

Plasma sample digestion

Aliquots of 50 µl of plasma were pipetted into a polypropylene 96-round well plate (Axygen, Corning, Tewksbury, MA, USA), after which the proteins were precipitated with 200 µl of methanol, followed by 1 min of high-speed vortex mixing and 10 min of centrifuging at 1500 × g. The supernatant was removed by inverting the plate above a waste vessel, and placing it upside down on a paper towel for 5 min.

A total of 50 µl of the internal standard working solution was added to each well, followed by 400 µl of a solution that contained the reagents required for the digestion: 50 mM ammonium bicarbonate buffer (pH ~8), 0.02% Tween-20 and 0.2 mg/ml trypsin in water, which was prepared directly before its addition to the sample. The plate was sealed and placed at 37°C while vortex mixing at 1100 rpm for 2 h.

Multidimensional SPE: extraction of the signature peptide from digested plasma

The extraction was performed using Oasis weak anion exchange (WAX) 30-mg cartridges with a 1-ml barrel size (Waters), which were conditioned with 1 ml of methanol and 1 ml of 50 mM ammonium acetate buffer (pH 5). The plasma protein digest (~450 µl) was mixed with 400 µl of 300 mM ammonium acetate buffer (pH 5) and loaded onto the cartridge. The cartridge was first washed with 1 ml of a solution, which contained 300 mM sodium chloride and 50 mM ammonium acetate buffer (pH 5) in water, followed by 1 ml of acetonitrile. Elution was performed using 1 ml of 1% aqueous ammonia. The eluate was transferred to a polypropylene 96-well plate and evaporated to dryness under a gentle stream of nitrogen at 60°C in approximately 90 min. The dried extract was reconstituted in 150 µl of a solution of 10% acetonitrile, 0.001% Tween-20 and 20 mM of ammonium acetate buffer (pH 5) in water, after which the plate was sealed and vortex-mixed for 1 min at 1100 rpm. Finally, the plate was placed in the autosampler for LC–MS/MS analysis.

LC–MS/MS Instrumentation

The LC system (ACQUITY I-class system [Waters]) comprised a binary LC pump, a vacuum degasser, a column oven (set at 45°C) and an autosampler (set at

10°C) equipped with a 50-µl loop extension. The analytical column was an ACQUITY CSH-C18 column (100 × 2.1 mm, 1.7 µm; Waters), which was operated at a flow rate of 0.400 ml/min. Gradient elution was performed with mobile phases consisting of 0.1% of formic acid in water (A) and acetonitrile (B). The gradient program ran from an initial 5% mobile phase B to 10% B in 6 min after which the system was washed at 90% B for 1 min, followed by equilibrating for 2 min at initial conditions before the next injection. The injection volume used was 15 µl.

Detection of the signature peptide and internal standard was performed with a Xevo TQ-S triple-quadrupole mass spectrometer (Waters) operated in positive electrospray ionization mode. The signature peptide was monitored using the SRM transition *m/z* 752.0 >773.3 while the transition *m/z* 756.0 >781.3 was used for the SIL internal standard. The following mass spectrometric settings were used: capillary voltage: 3000 V; desolvation temperature: 400°C; cone voltage: 45 V; and collision voltage: 25 V.

Method validation

The LC–MS/MS method was validated according to international guidelines [21–23] for bioanalytical method validation. In short, the accuracy and precision of the method were determined at four levels in sixfold in three analytical batches, performed on three separate days. Stability experiments were conducted for the intact protein as well as the for its signature peptide in all appropriate matrices and applicable experimental conditions. Blank plasma samples with and without the internal standard, a calibration curve and carryover blanks were measured in each analytical run. Recovery was evaluated and the influence of the matrix on the method was determined by the analysis of plasma samples prepared at the LLOQ of 10.0 ng/ml in six independent plasma lots. A more detailed description of the performed validation experiments can be found in section 1 of the Supplementary material.

Quantitative ELISA method

Costar® EIA/RIA half-well plates (Corning Inc., Tewksbury, MA, USA) were coated overnight with a monoclonal antibody, raised against the Nanobody. After removal of the coating solution, plates were blocked for 1 h with 0.1% casein in PBS. Samples, calibrators and quality control samples were transferred to the plate and incubated for 1 h at room temperature, while shaking at 600 rpm. Plates were washed three times with 0.05% Tween-20 in PBS, using an automated washer. Detection was performed with an inhouse produced biotinylated anti-Nanobody Nanobody for 30 min at room temperature. Streptavidin-labeled horse radish peroxidase was added and plates were incubated for 30 min, followed by a wash step. The coloring reagent 3,3'5,5'-tetramethylbenzidine was added and after 20 min at room temperature, the coloring reaction was stopped by addition of 1N hydrochloric acid, after which the plates were read at 450 nm with background subtraction at 620 nm using an ELISA reader (Tecan Group Ltd., Mannedorf, Germany).

Preclinical trial in rabbits & sampling

A preclinical trial was conducted in New Zealand white rabbits in order to determine the viability of intraocular dosing as a method of drug delivery. For this purpose, rabbits were given a single 50 µl intraocular dose of a 25 mg/ml solution of the Nanobody after which, at 2, 4, 8, 24, 48 and 96 h postdose, the aqueous humor and (citrate) blood samples were obtained. At each time point, three individual animals were sampled. The resulting plasma sample was transferred to a polypropylene tube, which was stored at -80°C until analysis. All animal tests were approved by the relevant ethical committee and the animal care and use committee at the contract research organization.

Results & discussion

Signature peptide selection

An *in silico* trypsin digestion of the Nanobody (a 28-kDa protein consisting of 259 amino acids) predicted 16 different peptides, 13 of which could be released from both identical subunits of which this protein consists. This is an advantageous feature, because the molarity of these 13 peptides after digestion is twice that of the protein prior to digestion, which facilitates detection. The online search algorithm Blast-P (version 2.2.28 [24]) was used to ascertain uniqueness of these peptides in both human and rabbit plasma. In rabbit plasma, all but one of the peptides were found to be unique, while in human plasma this was the case for only two of the peptides. The relatively high homology to proteins in human plasma displayed by this protein of camelid origin is explained by the fact it had been sequence optimized to reduce its immunogenic potential in humans. Peptides that were not unique in human plasma were rejected because method development was mainly performed in human plasma owing to the limited availability of rabbit plasma, and the potential to quantify the protein in human plasma in the future. Of the two remaining peptides, TFSYNPMGWFR (T3) and TGGSTYYPESVEGR (T7), the latter was selected as the signature peptide based on its superior selectivity in SRM mode compared with the other peptide candidate, as is shown in Figure 1. For both peptides, a considerable increase in background signal was found in the LC–MS/MS chromatograms for plasma,

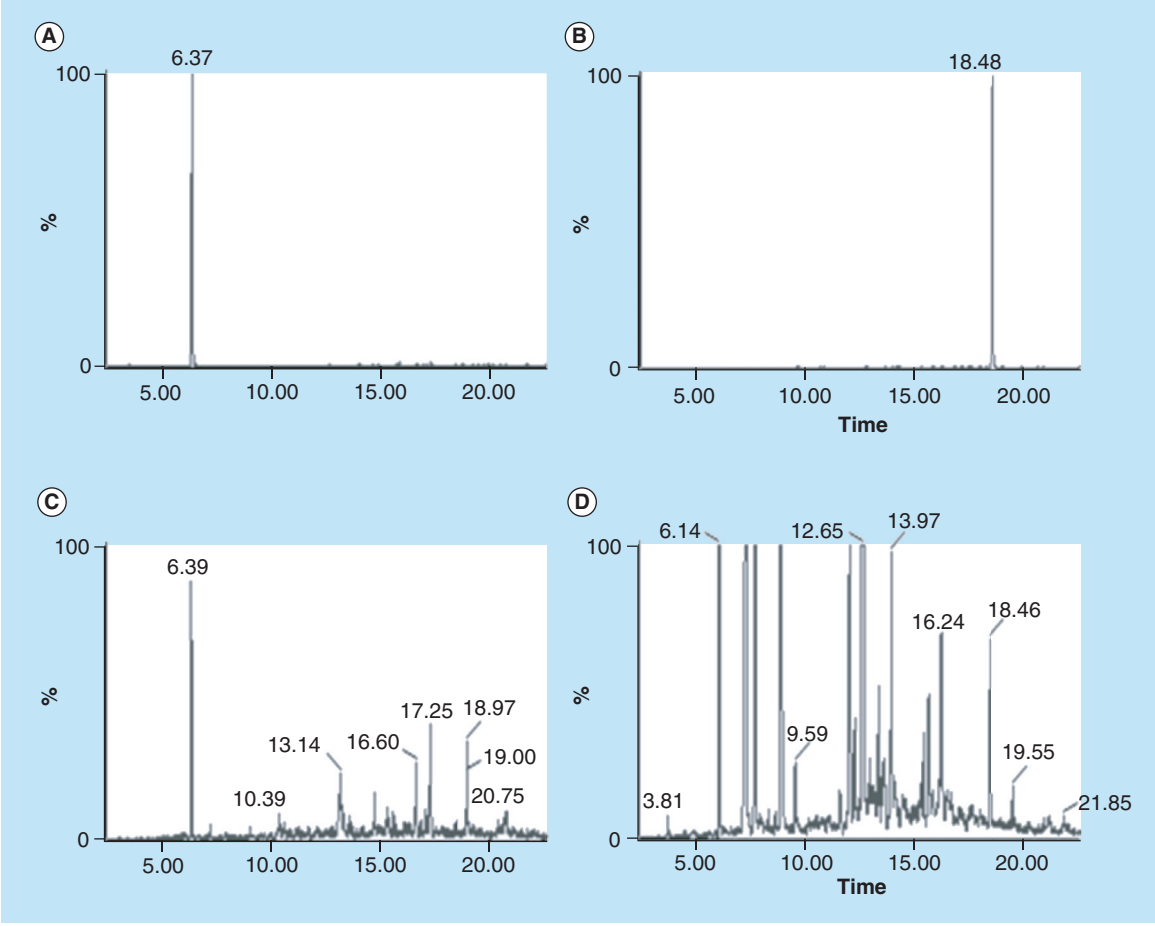

Figure 1. Evaluating matrix interferences during peptide selection. LC–MS/MS chromatograms (using nonoptimized LC conditions). Chromatograms of a digested buffer solution **(A & B)** and a digested plasma sample **(C & D)**, both of which contained 500 ng/ml of the biopharmaceutical. The SRM transition of the T7 peptide, which elutes at 6.4 min, is shown on the left **(A & C)** and the SRM transition of the T3 peptide, eluting at 18.5 min, on the right **(B & D)**. For each peptide the chromatograms were scaled to the intensity found in the buffered digest.

which was caused by the codigestion of plasma proteins to numerous peptides that produce interfering peaks, even in the highly selective SRM detection mode. For the T3 peptide, the effect is much larger than for the T7 peptide.

The signature peptide was monitored using the SRM transition *m/z* 752.0 > 773.3, which corresponds to the doubly charged peptide ion fragmenting to a singly charged y7 fragment. The internal standard, an inhouse prepared SIL version of the signature peptide in which four carboxyl ^{16}O-atoms were exchanged for their ^{18}O-isotopes, was monitored at *m/z* 756.0 >781.3, which corresponds to the doubly charged SIL peptide fragmented to its labeled, singly charged y7 fragment, on which all isotopes were retained.

Tryptic digestion

Direct tryptic digestion of plasma is unfavorable in a quantitative method for a number of reasons. Endogenous protease inhibitors such as α1-antitrypsin or α2-macroglobulin have an approximate plasma concentration of 1–2 mg/ml. This may reduce trypsin activity in a sample-dependent manner and thus lead to variability in digestion efficiency, therefore several common denaturing step such as the addition of guanidine hydrochloride, urea, deoxycholate or organic modifiers are currently in use [25]. Furthermore, plasma phospholipids that remain present in a plasma digest are known to cause ionization suppression in LC–MS/MS and may reduce the sensitivity of the method in a sample-dependent manner.

Both these situations can be avoided by the incorporation of a protein precipitation step and removal of the resulting supernatant before digestion [26]. The denaturing effect of the precipitant will deactivate proteins, including protease inhibitors, while the phospholipids are mostly removed along with the supernatant, as is known from the field of small molecule analysis [27,28]. For this reason, we decided to precipitate plasma proteins with methanol prior to trypsin digestion. A

time course of the digestion was recorded (Figure 2) based on which a digestion time of 2 h was selected for further experiments.

Internal standardization

The use of a proper internal standard is essential for the quantitation of proteins with LC–MS/MS. Several approaches exist, of which the use of a SIL form of the signature peptide combines good correction for experimental variability and ready availability for a reasonable price [29]. In particular, the preparation of an ^{18}O-labeled form of the signature peptide is a relatively fast and cost-effective means of obtaining an internal standard [19].

To generate a SIL internal standard, we performed an acid-catalyzed ^{18}O-exchange reaction. The peptide was dissolved in acidic $H_2{}^{18}O$, and stored at an elevated temperature, during which the ^{16}O-atoms of all carboxylic acid groups of the peptide were exchanged for the ^{18}O-atoms from the solvent. As shown in previous work [19], this is a generally applicable approach for small- to medium-sized peptides. However, the selected signature peptide is an especially good candidate for the creation of such an internal standard because it contains three carboxylic acids, the C-terminus and two glutamic acid residues with a total of six oxygen atoms, which results in an additional mass of 12 Da and for the doubly charged ion monitored an *m/z* difference of 6. Furthermore, the signature peptide contains no amino acids with amide groups, which may undergo deamidation, a known side reaction occurring under the conditions required for the exchange reaction that can lower the yield of the SIL internal standard. After acidic treatment of the signature peptide (*m/z* 752, MS spectrum in Figure 3A) in 1.5% HFBA for 47 h at 50°C, ^{18}O-exchange was found to be complete, and the internal standard formed had an *m/z* of 758 with no remaining unlabeled signature peptide at *m/z* 752 (Figure 3B).

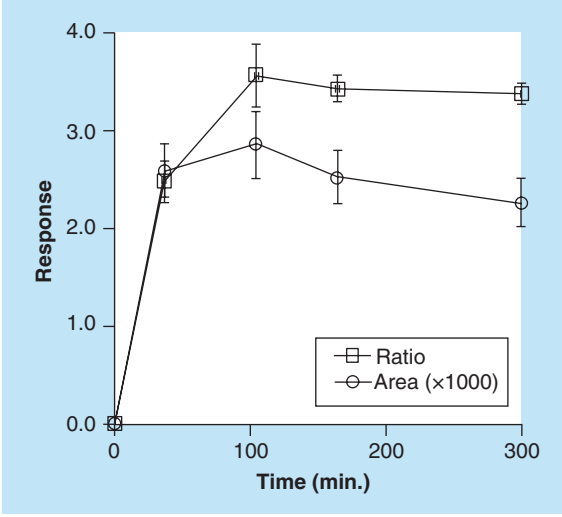

Figure 2. Time course of the digestion of the biopharmaceutical in plasma, expressed as the peak area of the signature peptide (counts ×1000) and the ratio of the peak area to that of the $^{18}O_4$-labeled internal standard.

Once incorporated into the carboxylic acid groups of a peptide, ^{18}O-atoms can be back-exchanged with ^{16}O-atoms when subjected to acidic conditions in an excess of normal water or, when they are present in the C-terminal carboxylic acid, if the peptide is exposed to active trypsin [30]. Since the sample will be exposed to normal water during sample preparation and LC–MS/MS analysis, acidic conditions and contact with active trypsin are to be avoided to maintain complete ^{18}O-labeling of the internal standard. This would mean that the internal standard needs to be added after the tryptic digestion has been completed and that no correction will be provided for the digestion step. To overcome these limitations we added the $^{18}O_6$-SIL internal standard to the sample before the digestion step, which allowed trypsin to completely remove the labeling from the C-terminal carboxylic acid, thus forming an $^{18}O_4$–$^{16}O_2$ peptide, which was monitored as an internal standard in the quantitative assay. Figure 3C shows the MS spectrum of the doubly charged ion of this peptide (at *m/z* 756) and demonstrates that neither the original, unlabeled signature peptide (*m/z* 752) nor the fully ^{18}O-labeled form (*m/z* 758) remained in the sample after this treatment. SRM analysis showed that the thus created $^{18}O_4$–$^{16}O_2$ internal standard contains less than 0.1% of the native $^{16}O_6$-signature peptide, which indicates an adequate level of isotopic purity (see section 5 of the Supplementary material). The pH of the solvents chosen for the subsequent extraction of the (labeled) signature peptide from the digest, as well as the pH of the reconstitution/injection solvent was set to a value of five or higher, which prevented acid-catalyzed back-exchange during sample processing or storage of the extract in the autosampler.

The presence of this internal standard during digestion offered correction for several sources of variability, such as chemical degradation or adsorption of the released signature peptide, Figure 2 shows that after approximately 100 min of digestion time, the peak area ratio remains constant while the peak area of the signature peptide shows a declining trend. This difference can be explained by the fact that both peptides, independent of their isotopic composition, will be equally subjected to any degradation or adsorption that might occur during the digestion. Even though the internal standard corrects for the decreasing peak area during the digestion process, a digestion time of 120 min was selected for the final method to limit this decrease to a minimum.

2D SPE

An important objective for any high-sensitivity LC–MS/MS method for protein quantitation in a biological matrix such as plasma is reducing the complexity of the digest. Although this can be achieved by using immunoaffinity-based extraction of either the protein [9,10] or the signature peptide [11,31], we preferred a more generic approach to avoid the analytical limitations associated with the use of antibody-based reagents [32–34]. This was accomplished by the application of multidimensional SPE to selectively extract the signature peptide from the mixture of digested plasma proteins. The SPE material used contains a mixed-mode WAX phase, capable of reversed-phase and anion-exchange interactions, both of which were used for optimum selectivity.

In the first separation dimension, the signature peptide was extracted from the digested plasma sample and retained by anion-exchange interaction. At pH 5, the three carboxylic acid groups of the signature peptide are negatively charged, while the tertiary amine group of the WAX phase bears a positive charge and thus captures the signature peptide along with all other anions in the sample. Since all tryptic peptides in the sample will have at least one C-terminal carboxylic acid group, little to no selectivity is introduced in this loading step. Undigested proteins, including trypsin, will, however, break through the cartridge unretained owing to size-exclusion effects.

Selectivity is introduced by exposure to a high ionic strength (0.30 M NaCl buffered at pH 5 with 50 mM acetate) during the first wash step. The high concentration of chloride ions causes the release and elution of peptides that are less strongly bound than the signature peptide, which contains three carboxylic acid groups. Although the nonprotic solvent acetonitrile in the second wash step is not capable of breaking ionic interactions, it will elute plasma constituents retained in reversed-phase mode, such as remaining (phospho)-lipids that were not completely removed during the protein precipitation step.

Further selectivity within the class of peptides was introduced by eluting with 1% ammonia (pH ~11), when the amine groups on the stationary phase become deprotonated and the anion-exchange interactions are broken. Relatively nonpolar peptides now remained bound to the cartridge by reversed-phase interactions, a retention mechanism that reportedly has a high orthogonality with the subsequent reversed-phase chromatographic separation at low pH [35]. The three charged carboxylic acid groups on the signature peptide increase its polarity at high pH, which causes the peptide to elute from the cartridge without the addition of an organic modifier to the elution solvent, thus separating it from more nonpolar peptides, which are retained on the cartridge. The presence of Tween-20 in the injection solvent did not result in a reduced sensitivity from ion suppression because the peptide eluted before the bulk of the Tween-20 polymer peaks, while its solubilizing effect on the peptide reduced adsorption problems after the SPE.

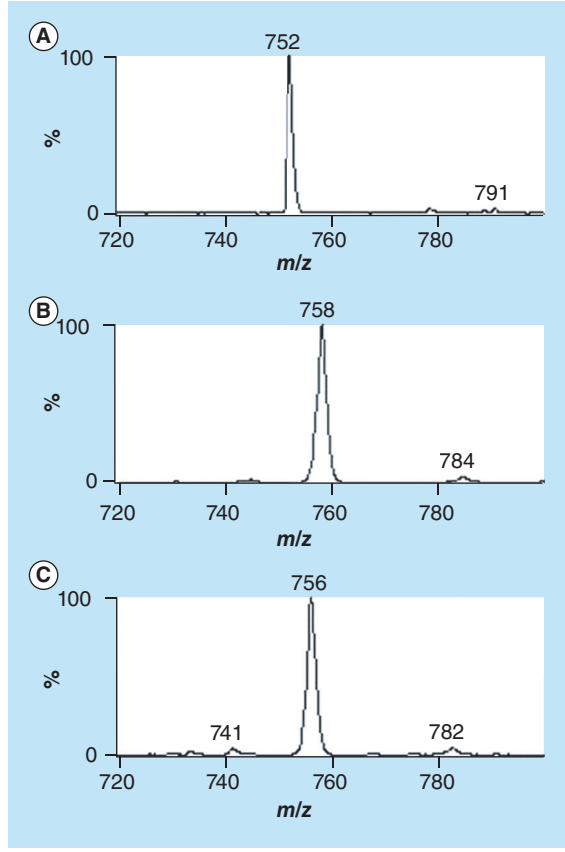

Figure 3. The changes in mass during the creation and use of the SIL-internal standard. (A) Q1 mass spectra for the doubly charged original $^{16}O_6$-signature peptide at an *m/z* of 752, **(B)** the $^{18}O_6$-internal standard formed after acid-catalyzed oxygen exchange at *m/z* 758 and **(C)** the $^{18}O_4$–$^{16}O_2$-form of the internal standard monitored in the quantitative assay, after enzyme-catalyzed back-exchange of the C-terminal oxygen atoms at 756 *m/z*.

The effect of this extraction procedure on the LC–MS/MS chromatogram is shown in Figure 4. Direct injection of a digested plasma sample resulted in a chromatogram in which a significant number of other peaks are present. After SPE, however, these peaks have largely been removed, which increases the sensitivity and reliability of the quantitation of the signature peptide.

Method validation in human plasma

The validation of the method was based on international guidelines for bioanalytical method validation

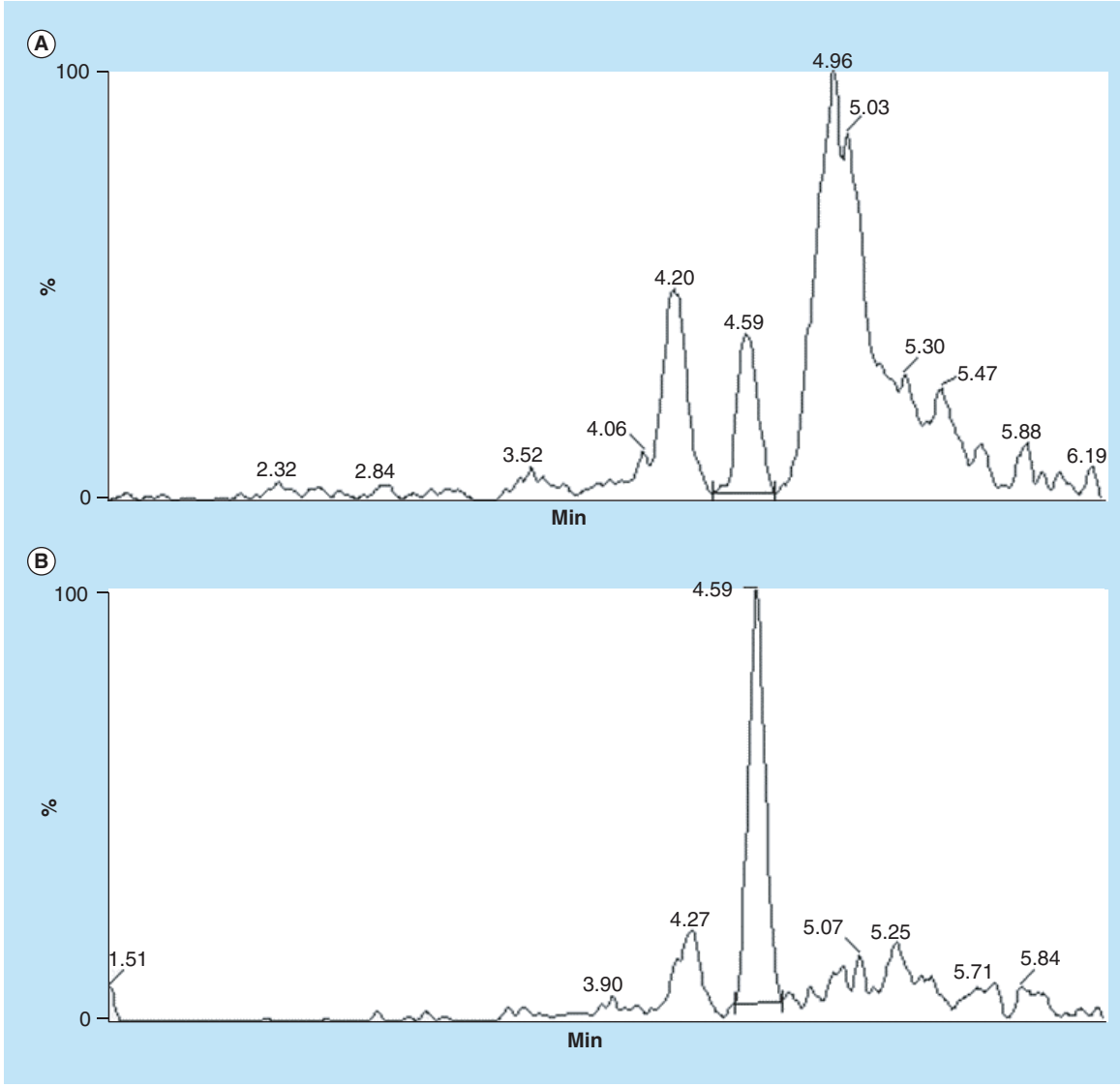

Figure 4. The effect of the SPE extraction. LC–MS/MS chromatograms in the SRM mode. Chromatograms of a digested plasma sample containing 10.0 ng/ml of the Nanobody® **(A)** before and **(B)** after the described SPE procedure. In both cases, the signature peptide elutes at 4.59 min.

and performed in citrated human plasma. The highest total bias and CV values obtained for each validation experiment are shown in Table 1, all of which are well within the acceptance limits of 15% (20% at the LLOQ) for small molecules [21–23]. A detailed description of the performed experiments can be found in section 1 of the Supplementary material and the obtained individual results are given in section 2.

The fact that the regular criteria for small-molecule validations were met indicates that the protein precipitation and trypsin digestion steps had been sufficiently optimized and did not introduce significant variability to the assay, even though the internal standard offered only partial correction for these steps.

The calibration curve was linear over the range of 10.0 to 10,000 ng/ml, and correlation coefficients (r^2) of 0.998 or higher were obtained in each run. Furthermore, the average deviation of the concentrations of the calibrators from the theoretical values did not exceed 2%. Compared with the ligand-binding assay, which uses a sigmoidal calibration curve, the linear calibration model has the advantage of a larger range, which reduces the amount of required dilutions of samples during bioanalysis.

The absolute recovery covering all the steps of the analytical procedure could not be determined because the analyte cannot be quantified without the digestion step. However, there are no set criteria for this parameter except that it should be consistent over the validated

concentration range. To demonstrate that this was the case, the correlation coefficient obtained in each separate validation run was calculated based on measured peak area and not the ratio, as the internal standard would correct for recovery inconsistencies during peptide extraction. The high calculated correlations indicated that there were no concentration-dependent influences from precipitation, digestion or peptide extraction, which would reduce the consistency of the recovery.

The stability of the intact protein was demonstrated in both plasma and the stock solution. The stability of the signature peptide and the internal standard were demonstrated in the autosampler stability validation experiment. For the internal standard, this demonstrates that the measures taken to prevent back-exchange were effective. By adding the internal standard before digestion, enzyme-catalyzed back-exchange during sample processing could no longer occur, as the ^{18}O-label was already removed from the C-terminal carboxylic acid, while acid-catalyzed back-exchange of the other carboxylic acid groups was prevented by the use of an injection solvent with a pH of 5.

Cross-validation to rabbit plasma

A limited, one-run, cross-validation to rabbit plasma was performed, which consisted of accuracy and precision samples prepared at four levels and analyzed in sixfold, and matrix variability samples spiked at the LLOQ in six independent rabbit plasma lots. For calibration, standards prepared in human plasma were used. The results of the cross-validation indicate that the method works equally well in human and rabbit plasma. A description of the cross-validation experiments can be found in section 1 of the Supplementary material and the obtained results are given in section 2.

LC–MS/MS analysis of rabbit plasma & aqueous humor samples

The plasma samples obtained after intraocular administration of the Nanobody to rabbits were analyzed using the described methodology. Owing to their higher concentrations, the aqueous humor samples were diluted with citrated human plasma by a factor of 10 before analysis. Rabbit citrate plasma was used as a dilution matrix to allow quantification against a plasma calibration curve and avoid the need to redevelop a separate method for aqueous humor. Figure 5 shows the pharmacokinetic curves obtained for both matrices and confirms that the LLOQ of 10.0 ng/ml was sufficient to support this preclinical trial. Calibration samples were prepared in human citrate plasma, while quality control samples were prepared in citrated rabbit plasma at three levels: 30.0, 800 and 8000 ng/ml. The individual calibration and quality control results can be found in section 3 of the Supplementary material.

A comparison of the results obtained by the current LC–MS/MS method to those obtained by a standard ELISA is shown in Figure 5 and Supplementary Figure 1 for both matrices. A good correlation between the ELISA and the LC–MS results was found throughout the sam-

Table 1. Maximal values for bias and CV observed for each of the performed validation experiments.

Validation item	Maximum bias (%)	Highest CV (%)
Method characteristics		
Accuracy and precision	3.2	5.4
Linearity	-1.1	5.4
Matrix variability	4.2	5.2
Carryover (% of response of the LLOQ)	2.7	N/A
Robustness	-1.6	5.6
Stability of the Nanobody® in plasma		
Benchtop (25 h and 50 h)	2.8	5.9
Freeze-thaw -20/-80°C (five cycles)	-14.6	5.5
Storage stability -20/-80°C (14 days)	-8	6.5
Stability of the Nanobody in stock solution		
Frozen storage -80°C (385 days)	-0.9	2.4
Benchtop (20 h)	2.3	4.1
Stability of the signature peptide in extracted plasma digest		
Autosampler 10°C (7 days)	-1.6	4.1

N/A: Not applicable.

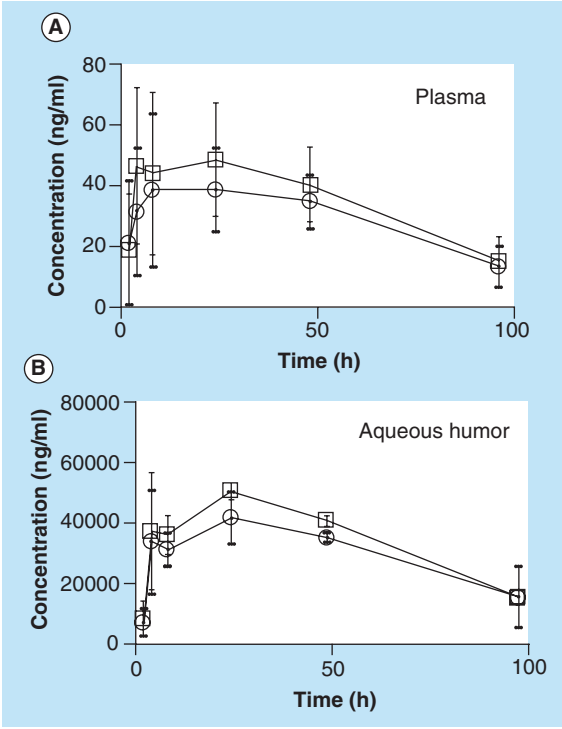

Figure 5. Concentration–time profiles of the Nanobody®. The profiles were measured by LC–MS/MS (squares) and ELISA (circles) in both plasma **(A)** and aqueous humor **(B)** after intraocular administration to rabbits (n = 3 animals per time point). The (biological) variation for each time point is expressed in the error bars.

pling period. Although slightly lower concentrations were typically measured with the ELISA, these differences were smaller than the observed biological variability between the animals for each time point. A possible explanation for the lower concentrations in the plasma samples as determined by ELISA is the potential binding of the analyte to its circulating target (von Willebrand factor) in the samples, which might reduce the analyte recognition of the assay or otherwise interfere with the ELISA.

Conclusion

In this work, we showed that by subsequent application of orthogonal dimensions of separation, sufficient selectivity can be introduced to allow high-sensitivity LC–MS/MS quantitation of a 28-kDa biopharmaceutical protein in complex biological samples without the use of immunocapture techniques. The developed procedure was successfully validated down to 10 ng/ml (0.3 nM) and applied to human and rabbit plasma.

An ^{18}O-labeled internal standard was readily prepared and offered full correction for all steps following the release of the signature peptide by trypsin during the digestion. The initial steps of the sample preparation procedure, protein precipitation and trypsin digestion, were therefore thoroughly optimized to reduce variability and maximize the response. This resulted in an analytical performance in accordance with international guidelines for validation and bioanalysis of small molecules.

The results obtained with the LC–MS/MS method were found to be in good agreement with the results from an ELISA method, which indicates the usefulness of the current LC–MS/MS method as an antibody-free alternative for high-sensitivity protein quantitation.

Future perspective

The use of LC–MS/MS for the absolute quantification of proteins is a relatively novel analytical platform for researchers in the bioanalytical field. It has clear analytical advantages compared with LBAs, including an extended linear range, typically better accuracy and precision through the use of SIL peptide internal standards, significantly reduced interference from cross-reacting molecules and protein–protein interactions, and independence from critical antibody-based reagents. Therefore, we expect that LC–MS/MS for protein quantification will continue to grow in importance in the next few years. In order to reach its full potential as an alternative bioanalytical platform, more research will be required to improve the concentration sensitivity of LC–MS/MS for protein quantification, which is currently still often inferior to that of LBAs. In this work, we provided an example of the potential of a generic, antibody-free approach for high-sensitivity protein quantification by LC–MS/MS, which we hope will contribute to the advancement of the field.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at www.future-science.com/doi/full/10.4155/bio.14.234.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Using 50 µl of human plasma, quantification of the 28-kDa Nanobody® down to 10.0 ng/ml was achieved without using immune enrichment.
- Careful optimization of the sample preparation conditions (proteolytic digestion, selective extraction and oxygen back-exchange prevention) was key in obtaining sufficient selectivity and sensitivity.
- The method was validated in human and rabbit plasma according to international bioanalytical guidelines.
- The method was successfully applied to quantify the Nanobody in plasma samples from a preclinical trial, and a good agreement was found with the results obtained by a standard ELISA.

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