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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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ABSTRACT

Background: 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.

Method: 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.

Results: 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 ($r^2 = 0.79$).

Conclusion: 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.

1. 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

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

Peptide sequence	Analyte	Used as	Precursor (m/z)	Product (m/z)	Product ion	Charge	CE[a] (V)	RF[b] (V)
APYTFGQGTK [$13C_6$,$15N_2$]	ADM	IS	539.27	746.38	y_7	1+	20	80
DILLTQSPAILSVSPGER [$13C_6$,$15N_4$]	IFX	Quantifier	953.53	555.25	y_5	1+	25	110
YASESMSGIPSR [$13C_6$,$15N_4$]	IFX	Qualifier	647.80	844.42	y_8	1+	25	90
ASQFVGSSIHWYQQR [$13C_6$,$15N_4$]	IFX	Qualifier	601.96	759.38	y_{12}	2+	15	80

[a] CE: Collision energy.
[b] RF: Radio frequency lens.

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.

2. Materials and methods

2.1. 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 (California, U.S.A). 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). Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Scientific (Waltham, MA). TPCK-Trypsin was supplied by Thermo Scientific as a lyophilized powder and was dissolved in acetic acid (50 mM) 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).

2.2. 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.

2.3. 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) coupled to a TSQ Quantiva, Thermo Scientific (Waltham, MA). 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). 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.

2.4. 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]. Fig. 1 displays the general principle of the method. The presence of NATI results in a reduction of SIL IFX signature peptide signal.

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 h 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 5 μL mixed working solution consisting of SIL ADM and SIL IFX (10 μg/mL) and gently vortexed for 5 min. 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 h 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 min 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 min to unfold the protein and allow for efficient digestion. Samples were centrifuged at 4000 g for 5 min 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).

2.5. 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

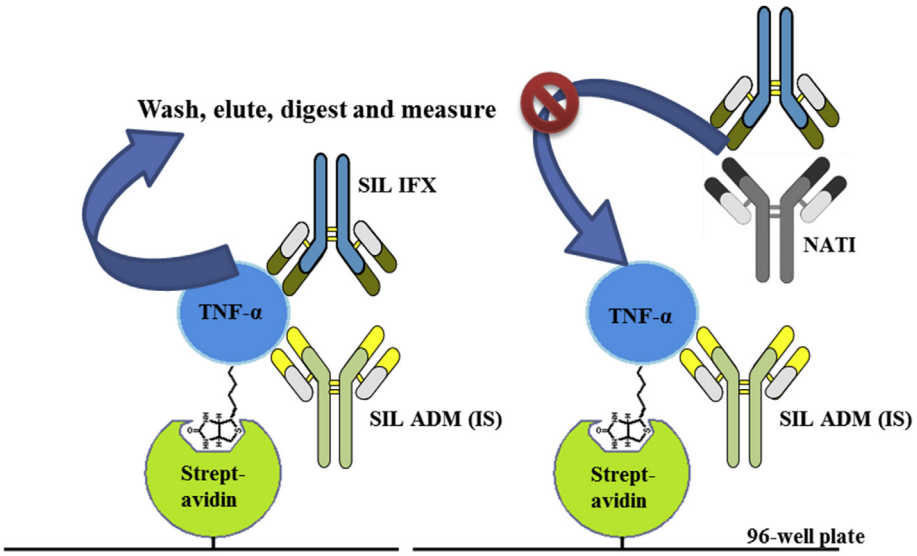

Fig. 1. Principle of the assay; In the absence of NATI in the sample, the maximum signal is measured (left). Lower SIL IFX signal is obtained proportional to the concentration NATI present in the sample (right). SIL ADM is used as internal standard to correct for loss during sample preparation and as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

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.

2.6. 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].

2.7. 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.

3. Results and discussion

3.1. 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 (80–300 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

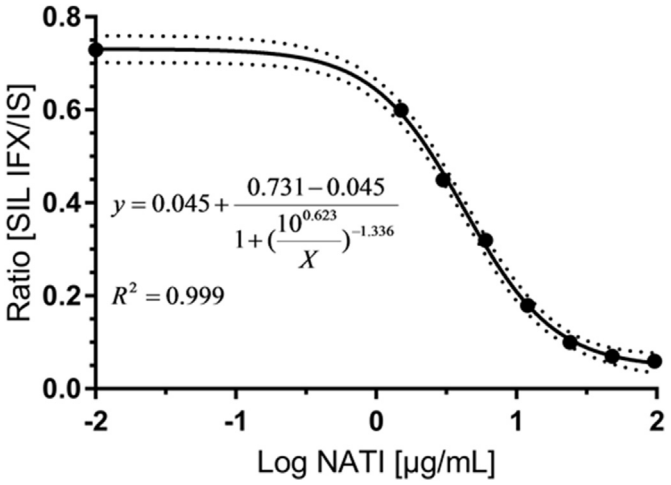

Fig. 2. Four-parameter logistic regression function was used for the NATI calibration curve. X-axis range (0 – 96 μg/mL) was converted to logarithmic scale, y-axis represents ratio IFX signal divided by IS (SIL AMD) signal.

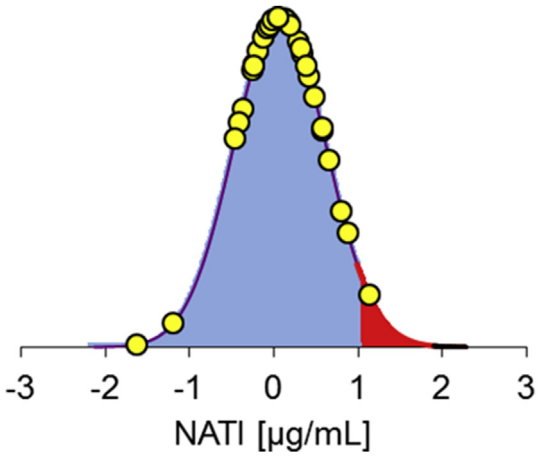

Fig. 3. Normal distribution of samples (n = 32) with no NATI, red border at 0.98 µg/mL represents Lc, black border at 1.89 represents Ld.

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].

3.2. Method evaluation

The calibration curve which had a range from 0–96 µg/mL NATI provided an excellent coefficient and determination ($R^2 = 0.999$). At 0 µg/mL NATI, the 5 µL 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). 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).

3.3. 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 $K_D = 1.8$ 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). 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

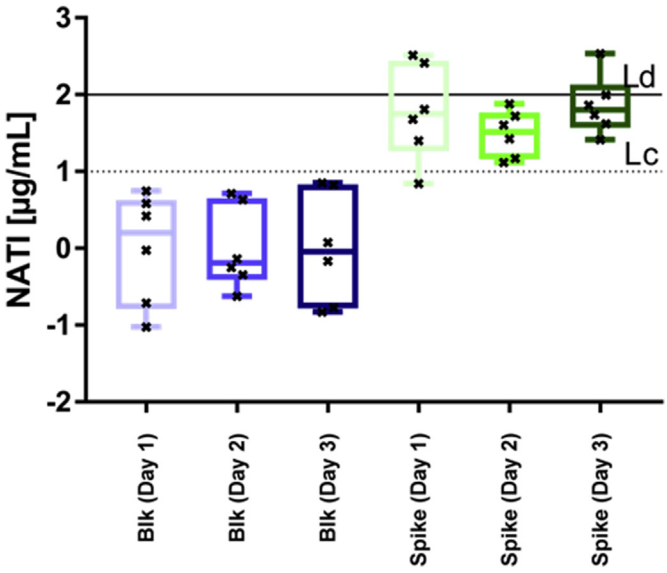

Fig. 4. Box and whiskers plot of the critical (Lc) and detection (Ld) levels. Data obtained from six individual human plasma samples never treated with infliximab (blk) and the same samples spiked with NATI (Spike). Test performed on 3 different days.

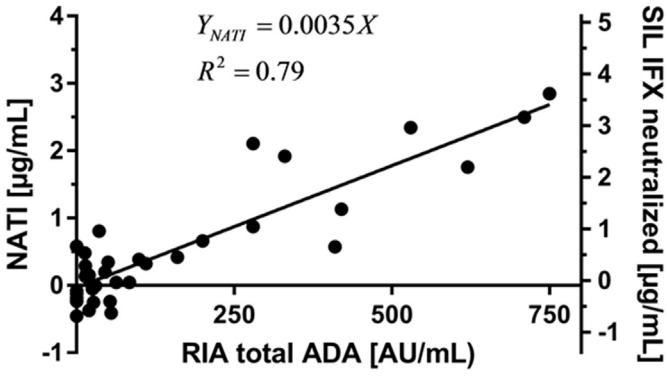

Fig. 5. Total ADA versus NATI (lefty-axes) and SIL IFX neutralized (right y-axes), n = 36, levels >880 AU/mL were not included.

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 0–3.8 µg 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 and 96 µg/mL NATI.

4. Conclusion/discussion

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 (<1 μg/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 and 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 (1 μg) 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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