Evaluation of the potential use of hybrid LC–MS/MS for active drug quantification applying the ‘free analyte QC concept’

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Aim: Assessment of active drug exposure of biologics may be crucial for drug development. Typically, ligand-binding assay methods are used to provide free/active drug concentrations. To what extent hybrid LC–MS/MS procedures enable correct ‘active’ drug quantification is currently under consideration.

Experimental & results: The relevance of appropriate extraction condition was evaluated by a hybrid target capture immuno-affinity LC–MS/MS method using total and free/active quality controls (QCs). The rapid extraction (10 min) provided correct results, whereas overnight incubation resulted in significant overestimation of the free/active drug (monoclonal antibody) concentration. Conventional total QCs were inappropriate to determine optimal method conditions in contrast to free/active QCs. **Conclusion:** The ‘free/active analyte QC concept’ enables development of appropriate extraction conditions for correct active drug quantification by hybrid LC–MS/MS.

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Detailed characterization of the pharmacokinetics (PK) and pharmacodynamics (PD) of drug candidates plays a basic role in the drug development process. Various analytical techniques can be used to provide quantitative data to assess drug exposure and for PK calculations. However, depending on the technology, assay format and specific procedure applied, the concentration of either the total drug, the target-binding competent drug or the active drug can be quantified [1]. In cases, when a therapeutic protein drug is administered at a high concentration and/or the concentrations of soluble ligand or shed receptor or antidrug antibody are low, the determined free/active concentration is approximately equal to the total drug concentration. However, the situation can be, and often is, much more complex if the drug is administered at lower concentrations or if the ligand, shed receptor or antidrug antibody are present at concentrations that lead to substantial amounts of complexed and thus neutralized drug, resulting in reduced active-drug exposure. Since the active drug is considered to be the most relevant species in obtaining a meaningful understanding and interpretation of PK and PD data, active drug assays have become very important at all stages of drug development. For example, in the absence of an appropriate PD marker, active drug assays are crucial, since the validity of nonclinical safety studies relies upon the demonstration of active drug exposure over the whole dosing phase of the study [2].

Ligand-binding assays (LBA) are the standard technology for bioanalysis of therapeutic protein drugs owing to their excellent sensitivity, selectivity, high throughput and the ability to detect either the total or active drug concentration [3–7].

However, it has been recognized that accurate quantification of the absolute active drug concentration within a mixture of drug–ligand complexes, without perturbing the equilibrium and thus biasing the correctness of the determined active drug concentration, is challenging [5,6]. For accurate free drug quantification, the selection of an appropriate assay format and assay reagents is crucial, which has been addressed and discussed in the literature [4–6]. Using a ligand as the capturing reagent is the favored option as it provides the best representation of the drug–ligand

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interaction. Alternately, an anti-idiotypic antibody as the capturing reagent in the LBA could be used in cases where the ligand is not available in the required quality or the drug–target/ligand interaction has unpropitious rate constants (e.g., a slow association rate with a high dissociation rate). If an anti-idiotypic antibody is selected, a careful assessment in the characterization of the reagent should be applied, especially whether it really competes with the same epitope as the target [7]. Main focus should be whether the anti-idiotypic antibodies really detect only free drug molecules and not drug–ligand complexes, or to what extent it perturbs the equilibrium, for example, because it has a higher affinity toward the drug than the ligand or to direct competition with the ligand in the drug–ligand complex.

In recent years, LC–MS/MS has evolved into a promising and complementary technique for the quantification of therapeutic protein drugs as well as protein biomarkers and other ligands in complex biological matrices, such as serum and plasma. The ‘bottom-up’ approach, where the sample including the therapeutic protein is converted into peptides by an enzymatic digestion followed by quantification of the drug via a specific signature peptide [8–11], is considered to provide ‘total drug’ data. However, antidrug antibodies have been reported to impact on correct drug quantification and thus need to be considered for appropriate method development [12,13].

To improve the sensitivity of an assay, interference of sample matrix can be reduced via immunoaffinity enrichment of the analyte using analyte-specific ligands or antibodies. For therapeutic monoclonal antibodies, protein A/G or antihuman IgG antibodies could be used as a generic enrichment procedure. This type of hybrid LC–MS/MS assays, combining multiple reaction monitoring detection with immunoaffinity enrichment, improves the sensitivity and specificity of protein quantification. Furthermore, the use of a drug-specific capturing step (e.g., using the ligand or an anti-idiotypic antibody) in a hybrid LC–MS/MS method has been claimed to enable free/active drug quantification [14].

However, a detailed assessment of the critical factors, such as ligand interference, incubation time and sample dilution [5,6], has not been carried out by the authors to test the potential influence of the method procedure on the equilibrium of the drug and its binding partners, and thus on the accuracy of the provided results.

An appropriate calibration and quality control (QC) concept is, however, crucial for the qualification and validation of free/active drug assays. First, the use of a ligand-free calibration matrix is a fundamental prerequisite [6,15,16], which is often overlooked. Second, QC samples prepared in the conventional way (with total drug), as described in the guidelines and white papers [8,17–20], are not suitable for assessing the potential influence of the applied sample treatment on the drug–ligand equilibrium, and thus the use of such QC samples for correct and accurate ‘free drug’ data quantification is questionable.

We introduced the ‘free analyte QC’ concept for appropriate free/active drug method development and validation [16]. This concept is based on QC samples containing exactly defined amounts of free drug in equilibrium with the respective drug–ligand complex and enables evaluation of any factors that may influence this equilibrium. The concept has been successfully applied in the LBA field [15].

In this publication, we describe the use of the ‘free analyte QC’ concept for the evaluation of the potential and limitations of hybrid LC–MS/MS methods using target-capture for correct active drug quantification. Particular attention is paid to the importance of an appropriate extraction procedure, especially the incubation time, and to discussing the advantages and disadvantages of the use of conventional ‘total QC’ and ‘active QC’ samples. The ‘active QC’ samples contained a defined amount of active drug. In case of the used model compound, a therapeutic monoclonal antibody (mAb <X>) that binds a soluble target X, active drug is defined as all molecules with at least one free binding site (‘total free’ and ‘partially free’ mAb <X> [Figure 1](#)).

In addition, the Gyrolab technologyTM (Gyros AB, Uppsala, Sweden) was applied as an orthogonal LBA to confirm the correctness of the active QC samples and that the hybrid LC–MS/MS method was correctly quantifying the active drug concentrations.

Experimental Materials

A therapeutic monoclonal antibody (mAb <X>, humanized IgG1), ligand protein ‘X’ (recombinant, human) and biotinylated ligand protein (capture molecule) were provided by Roche Diagnostics GmbH (Penzberg, Germany). Alexa647 labeled antihuman Fc antibody was produced by using the labeling kit from Molecular Probes (OR, USA, Kit: A20173) and following the suppliers instructions. Universal buffer for ELISA (ready to use), phosphate-buffered saline (PBS), streptavidin-coated magnetic beads (MBs, 20 mg/ml), Tween-20 and dithiothreitol were provided by Roche Diagnostics GmbH. As wash buffer for the LBA and the MB extraction approach, a PBS solution containing

Figure 1. Schematic illustration of the active and inactive drug fractions.

The monoclonal antibody can form complexes with one ligand (red circle) bound (partial free) or two ligands bound (total bound). Total and partial free antibody combined is considered to be the active drug.

0.05% Tween-20 was used. Blank pooled horse serum was purchased from PAN Biotech (Aidenbach, Germany). Iodoacetamide, urea and formic acid were purchased from Sigma-Aldrich (MO, USA). Ammonium bicarbonate was purchased from AppliChem GmbH (Darmstadt, Germany). Sequencing-grade trypsin was obtained from Promega (WI, USA). Ultrapure water was produced in an arium[®] 611 device from Sartorius Weighing Technology GmbH (Goettingen, Germany). Bioaffy[™] 200 CDs were purchased from Gyros[™] (Gyros AB, Uppsala, Sweden). Tubes for incubation were used from Eppendorf (Hamburg, Germany) at a volume of 1.5 ml and conical microtiter plates were purchased from Gyros. All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Gyrolab[™] immunoassay format

Calibration standards and active QC samples were diluted tenfold in assay buffer. Biotinylated ligand protein was used as the capture reagent and the antihuman Fc monoclonal antibody labeled with Alexafluor 647 was used for detection. Capture and detection reagents were diluted to 10 and 0.5 µg/ml, respectively, in assay buffer. All assay components were placed in conical 96-well polypropylene microtiter plates and then sealed with adhesive foil (Thermo Scientific, PA, USA) prior to loading into the instrument. The assay components were loaded onto streptavidin beads inside the micro-column structures of Bioaffy 200 CDs (Gyros AB) by the Gyrolab system, beginning with the capture reagent, followed by the calibrator or QC sample and finally the detection reagent. After each step the beads were washed with washing buffer.

Hybrid LC–MS/MS assay format

First, the biotinylated ligand protein was immobilized onto MBs. A 1-ml aliquot of MBs was washed twice with 1 ml of assay buffer. Biotinylated receptor protein (19.4 µg) in 1 ml of assay buffer was incubated with MB for 16 h at room temperature. Subsequently, the MBs coupled with biotinylated receptor protein were washed three-times with 1 ml of washing buffer. The ligand-immobilized beads were then stored until required for analysis at 4°C in 1 ml of washing buffer.

Prior to quantification of active antibody by immunoaffinity LC–MS/MS, sample processing and MB separation were performed using a DynaMag 2 magnet separator (Invitrogen, Oslo, Norway). For the washing and incubation steps, the sample tubes were removed from the magnet.

Blank sample, calibration standards, active QC samples and total QC samples (details in the 'Preparation of total and active QC samples' section) were prediluted twofold in assay buffer, resulting in a serum concentration of 50% to minimize the occurrence of dilution-induced complex dissociation and were immediately further processed.

MB-immobilized ligand protein (200 µl) was added to a sample tube (Eppendorf) and the supernatant was removed using a magnetic separator, then a 100-µl aliquot of prediluted sample was added to the tube.

The sample tubes were incubated for 10 min or overnight at room temperature with shaking at 600 rpm to capture active mAb < X>. After removing the supernatant, the beads were washed twice with 200 µl of washing buffer and once with 200 µl of PBS. Subsequently, 50 µl of 800 ng/ml SILu[™] MAb (a stable isotope-labelled

[SIL] peptide; Sigma-Aldrich, MO, USA) as the internal standard (IS) in 50 mM ammonium bicarbonate/6 M urea/5 mM dithiothreitol was added to each tube, then the tubes were incubated for 1 h at 37°C. Next, 20 µl of 50 mM iodoacetamide in 50 mM ammonium bicarbonate was added and the tubes were incubated for 30 min at 37°C in darkness. Subsequently, 250 µl of 4 µg/ml trypsin in 50 mM ammonium bicarbonate was added and the tubes were incubated at 37°C overnight. Finally, the supernatant in each tube was transferred to a glass vial (Waters Corporation, MA, USA) followed by adding 5 µl of 10% formic acid to deactivate any residual trypsin. The resulting trypsin-digested samples were then analyzed by LC–MS/MS.

LC–MS/MS instrumentation & conditions

The tryptic peptides in the digested sample were separated using an Agilent 1100 series LC assembly consisting of a G1312A binary pump, a G1313A autosampler and a G1316A column compartment set to 50°C (Agilent, Waldbronn, Germany).

A 20-µl aliquot of sample was injected onto an Eclipse XDB-C18 analytical column (5 µm particle size, 150 × 4.6 mm i.d.; Agilent) using the following mobile phases at a flow rate of 300 µl/min: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. From 0 to 1 min, the solvent composition was held at 15% B and the gradient started at 1 min increasing to 70% B at 15 min, then rapidly increasing %B to 90% over 0.1 min, maintaining %B at 90% for 1.9 min and then decreasing it back to 15% at 17 min, followed by the system equilibration until 25 min.

Mass spectrometric analysis was performed using a TSQ Quantum UltraTM triple quadrupole mass spectrometer using electrospray ionization in the positive ion polarity mode with an electrospray source (Thermo Fisher Scientific, CA, USA). The mass spectrometer was operated in MRM mode. The conditions in the ionization source were spray voltage of 5000 V and capillary temperature of 190°C. The following transitions were monitored: for GPSVFPLAPSSK (a surrogate peptide of our mAb <X>) Q1 593.8 [M + 2H]²⁺ to Q3 699.4 (y71⁺) and for the SIL peptide (surrogate peptide of SILuMAb [IS]) Q1 597.8 [M + 2H]²⁺ to Q3 707.4 (y71⁺). The optimized collision energies for GPSVFPLAPSSK and the SIL peptide were 20 V and 2.5 mTorr (collision gas), respectively. The peak width for Q1 and Q3 was set to 0.9 (FWHM). The scan width was 1 m/z and the scan time was 100 m/s.

Calibration standards

For calibration, a target free/noncross-reactive horse serum was used as a surrogate matrix. The calibration standards were prepared by diluting the mAb <X> in serum, resulting in total mAb <X> concentrations of 0.313, 0.625, 1.25, 2.50, 5.00, 10.0 and 20.0 µg/ml. These calibrants were used for both the LC–MS/MS and the Gyros assays to easily enable a comparison of the results from both methods. It should be noted that the Gyros assay is significantly more sensitive than the LC–MS/MS assay, but in this work it was applied as an orthogonal method to confirm the LC–MS/MS results, so sensitivity was not our main focus. Thus, the Gyros assay was not optimized to be sensitive, rather to be capable to measure the identical calibrators with low minimum required dilution.

Comparability of assay formats

For both the hybrid LC–MS/MS and Gyros methods, the same reagent (biotinylated ligand) was used for capturing the mAb <X>. The ligand capture approach only captures active mAb (X; total free and partially free drug) but not inactive drug (total bound drug; see Figures 1 & 2). In both assays a 'generic' IgG detection is performed, either by an antihuman Fc antibody or by a human IgG-specific signature peptide.

Preparation of total QC samples

The total QC samples were prepared by spiking horse serum with mAb <X> at 0.4, 1, 1.2, 3, 5, 12.5, 32 and 80 µg/ml. The QC samples at 0.4 to 12.5 µg/ml were within the calibration range. The total QCs at 32 and 80 µg/ml were above the calibration range and were only used to prepare active QC samples with a concentration within the assay range (see in the 'Preparation of free/active QC samples' section). The total QC samples at 0.4–12.5 µg/ml were used to prove the precision and accuracy of the LC–MS/MS analytical method.

Preparation of free/active QC samples

The active QC samples were prepared by mixing ligand and mAb <X> in defined ratios (Figure 3 & Table 1) to generate active mAb <X> concentrations containing 20 or 50% active drug (i.e., with at least one free binding

Figure 2. Assay formats: free/active therapeutic monoclonal antibody captured by a biotinylated ligand immobilized on a streptavidin surface and quantitated in proportion to fluorophore intensity (Gyrolab™ immunoassay) or signature peptide abundance (hybrid LS-MS/MS assay). (A) Assay based on consecutive steps of capturing active mAb <X> and generic readout with an antihuman Fc detection antibody labeled with Alexa 647. (B) LC-MS/MS assay based on consecutive steps of capturing active mAb <X> (10 min or ON), denaturation, reduction, alkylation and digestion. Quantification of the active drug is based on a selected generic human IgG-specific peptide. Ab: Antibody; mAb: Monoclonal antibody; ON: Overnight.

site). The samples were incubated overnight to ensure that equilibrium was reached between the drug and ligand. A total of eight QC samples prepared in the preceding section were used to generate four pairs of active QC samples by addition of defined amounts of the ligand to result in either 50 or 20% active drug. Each pair of active QC samples contained identical active drug concentrations but different total drug concentrations.

The required ligand concentration for each active QC sample was calculated according to Equations 1, 2 and 3 using the equilibrium constant K_D .

$$K_D = \frac{[A_{\text{free}}] \cdot [B_{\text{free}}]}{[AB]} \quad \text{Equation 1}$$

$[A_{\text{free}}]$ is the free ligand concentration ($= [A_{\text{total}}] - [AB]$), $[B_{\text{free}}]$ is the free mAb <X> concentration ($= [B_{\text{total}}] - [AB]$) and K_D is the equilibrium constant between the mAb <X> and ligand.

$$B_{\text{free fraction}} = [B_{\text{free}}]/[B_{\text{total}}] = \left\{ \frac{[B_{\text{total}}] - [A_{\text{total}}] - K_D}{2} + \sqrt{\frac{([A_{\text{total}}] + [B_{\text{total}}] + K_D)^2}{4} - [A_{\text{total}}] \cdot [B_{\text{total}}]} \right\} / [B_{\text{total}}] \quad \text{Equation 2}$$

Equation 2 was used for a one-to-two interaction, where two ligands bind to a bivalent mAb. For a bivalent therapeutic antibody, the concentration of the antibody is converted in concentration valences by multiplying the mAb <X> concentration by a factor of 2. Each $[B_{\text{free}}]$ was calculated by using the total ligand concentration and the total mAb <X> valence concentration. The free fraction of B was calculated by dividing $[B_{\text{free}}]$ by the total amount of B ($[B_{\text{total}}]$), in our case the total mAb <X> concentration. To convert the calculated B_{free} fraction into the active mAb <X> concentration, Equation 3 was used. Equation 3 combines the probability of the presence of mAb <X> in partial free and total free in the active mAb <X> concentration.

Figure 3. Scheme for the preparation of 50 and 20% free/active drug quality controls by incubating total quality controls with a recombinant human ligand. Total eight QC samples (QC: 1–8) were pairwise transformed to active QC samples either containing 20 or 50% active drug by adding human ligand to the QCs. Total QCs 1–6 (concentration 0.4–12.5 µg/ml) and active QCs 3–8 (with active concentrations of 0.6, 2.5 and 16 µg/ml) fall within the calibration range of the LC–MS/MS assay. LBA: Ligand-binding assay; QC: Quality control.

Table 1. Accuracy and precision of total drug determinations by LC–MS/MS after a 10-min or overnight immunoaffinity enrichment.

Active drug conc. (µg/ml)	Reduction of total drug (%)	Added drug conc. (µg/ml)	Added ligand conc. (nM)	Hybrid LC–MS/MS						Gyros		
				10-min incubation intrabatch (n = 3)			ON incubation intrabatch (n = 3)			Intrabatch (n = 3)		
				Mean	%Accuracy	%CV	Mean	%Accuracy	%CV	Mean	%Accuracy	%CV
0.20	-50	0.4	3.9	BLQ [†]	–	–	0.50	251	1 [‡]	–	–	–
0.20	-80	1.0	12.4	BLQ	–	–	0.71	354	6 [‡]	–	–	–
0.60	-50	1.2	11.4	0.48	80 [‡]	16 [‡]	1.07	178	17 [‡]	0.62	103 [‡]	1.3 [‡]
0.60	-80	3.0	36.0	0.68	113 [‡]	2 [‡]	1.66	277	16 [‡]	0.65	109 [‡]	1.1 [‡]
2.50	-50	5.0	46.8	2.94	117 [‡]	6 [‡]	3.86	154	19 [‡]	2.46	99 [‡]	1.4 [‡]
2.50	-80	12.5	148	3.00	120 [‡]	2 [‡]	5.98	239	22	2.38	95 [‡]	0.3 [‡]
16.0	-50	32.0	298	19.0	119 [‡]	3 [‡]	ALQ [§]	–	–	15.5	97 [‡]	3.1 [‡]
16.0	-80	80.0	944	18.5	116 [‡]	12 [‡]	ALQ	–	–	14.4	90 [‡]	1.5 [‡]

[†]Below the lower limit of quantification (<0.313 µg/ml).
[‡]Met the acceptance commonly used by LBAs (%Accuracy: 80–120%, %CV: <20%).
[§]Above limit of quantification (>20 µg/ml).
ALQ: Above limit of quantification; BLQ: Below the lower limit of quantification; Conc.: Concentration; CV: Coefficient of variation; ON: Overnight; QC: Quality control.

$$\text{active_mAb(X)} = \left\{ \left[\text{Bfreefraction} \right]^2 + 2 \cdot (1 - \left[\text{Bfreefraction} \right]) \cdot \left[\text{Bfreefraction} \right] \right\} \cdot \left[\text{Btotal} \right]$$
 Equation 3

As an example:

One active QC sample with 0.6 µg/ml mAb <X> was produced by mixing 3 µg/ml mAb <X> (~40 nM valence) with 36 nM ligand. This resulted in approximately 11% free fraction, which was converted to an active concentration of 0.6 µg/ml using Equation 3.

The aim in this work was to perform a principle evaluation of the influence of the applied assay procedure on the equilibrium. The QC ranges were selected to fall within the assay range of the hybrid LC–MS/MS method to enable the technological assessment; the biologically relevant ligand concentrations were not considered.

Data processing

For the Gyrolab immunoassay, signals were obtained at the 5% photomultiplier-tube level using the Gyrolab Evaluator Program. For the LC–MS/MS assay, LCQUAN™ version 2.0.7 (Thermo Fisher Scientific) was used for the data processing.

Since the use of SILuMab as an IS did not provide any improvements in the accuracy and precision of the LC–MS/MS data (data not shown) compared with the external calibration method, the drug concentrations were calculated by the external calibration method using the selected peptide of the mAb <X> [21]. Standard curves were obtained by plotting the signal or peak area versus the mAb <X> concentration (ng/ml) for the Gyros immunoassay or LC–MS/MS assay, respectively. QC recoveries were calculated using XLFit (version 5.3.1.3, IDBS Ltd, Guildford, UK) with a sigmoidal (Rodbard_205) curve fit using weighting by signal for the Gyros assay and the model equation 606 with weighting by area for the LC–MS/MS. The back-calculated concentrations of the calibration standard were within ±15% of the nominal concentration.

Results & discussion

The introduction of a drug-specific capture step prior to mass spectrometric analysis is considered to be a viable option to enable free/active drug quantification, even with bottom-up mass spectrometric methods [14]. The use of a ligand-binding step in the LC–MS/MS hybrid assay procedure means, however, that the analytical challenges and considerations that are encountered with active drug LBAs are also relevant to hybrid MS methods. Independent of the technology used, the application of appropriate ligand-free calibration standards is a fundamental prerequisite to avoid any calibration-induced bias in the results, as discussed in our previous work [6,15,16]. The overriding technical challenge is to minimize any shift in the equilibrium between the drug and its binding partners.

Sample dilution and the selected dilution medium can have a significant impact on the accuracy of the result. The use of a ligand-free dilution medium results in the dissociation of existing drug–binding partner complexes and consequently, to an erroneous increase in the determined active drug concentration. Conversely, the use of a ligand-containing medium erroneously reduced the active drug concentration since additional ligand is added with each dilution step, which can then form complexes with the initially present free/active drug molecules [6]. Furthermore, the reagent that is used to perform the ligand-binding extraction step could also interfere with the equilibrium and thus, bias the results.

The focus of the current investigation was to better understand the potential and limitations of target-capture hybrid LC–MS/MS methods for correct active drug quantification, focusing on the importance of the ligand-binding step of the procedure.

For the evaluation, a target capture hybrid LC–MS/MS method was developed for the quantification of a mAb <X>, a humanized IgG1 with a soluble target.

The ligand-binding extraction was performed using a bead-based procedure with the recombinant ligand used as the capture reagent. The LC–MS/MS part of the procedure was performed using a well-described bottom-up approach with GPSVFPLAPSSK as a signature peptide derived from the CH1 domain of humanized IgG1 after tryptic digestion. This generic peptide has been used for the quantitative bioanalysis of humanized therapeutic monoclonal antibodies in preclinical species [22–24].

In addition to the hybrid LC–MS/MS procedure, a Gyros-based LBA method was used as an orthogonal technique. Schematics of the assay formats are provided in Figure 2.

The evaluation was performed using a panel of total and active QCs, which were prepared in horse serum as a ligand-free matrix.

Eight concentration levels of ‘total’ QCs (0.4, 1.0, 1.2, 3.0, 5.0, 12.5, 32 and 80 µg/ml; Total QCs: 1–8 in Figure 3, respectively) were prepared according to the current guidelines and were used for method development (QCs: 1–6 at 0.4–12.5 µg/ml were within the assay range).

Table 2. Accuracy and precision of free/active drug determinations by LC–MS/MS and Gyros-based immunoassay.

Total drug conc. (µg/ml)	10-min incubation intrabatch (n = 3)			ON incubation intrabatch (n = 3)		
	Mean	%Accuracy	%CV	Mean	%Accuracy	%CV
0.40	0.43	107 [§]	11 [§]	0.43	108 [§]	6 [§]
1.00	1.15	115 [§]	4 [§]	0.80	80 [§]	13 [§]
1.20	1.19	100 [§]	19 [§]	1.23	102 [§]	20 [§]
3.00	2.54	85 [§]	13 [§]	2.52	84 [§]	9 [§]
5.00	4.00	80 [§]	16 [§]	3.94	79	6 [§]
12.5	13.7	109 [§]	16 [§]	10.6	85 [§]	7 [§]
32.0	ALQ [†]	–	–	ALQ [†]	–	–
80.0	ALQ [†]	–	–	ALQ [†]	–	–

[†] Above limit of quantification (>20 µg/ml).
[§] Met the acceptance criteria commonly used by LBAs (%Accuracy: 80–120%, %CV: <20%).
 ALQ: Above limit of quantification; Conc.: Concentration; CV: Coefficient of variation; LBA: Ligand-binding assay; ON: Overnight; QC: Quality control.

To investigate the potential impact of the overall sample analysis, workflow on the equilibrium of the drug and its soluble ligand, active QCs were prepared as described in our previous work [16]. These active QCs were prepared by addition of defined amounts of the ligand to the ‘total’ QCs, followed by calculation of the active drug concentration (total free and partially free) using the binding affinity data. These calculated active drug concentrations are then set as the target values for the active QCs. To assess the role of the drug/ligand ratio in a sample in the perturbation of the equilibrium, QCs with identical active drug concentrations but at two different ratios (50 and 20% active drug with 50 and 80% total bound drug, respectively) were prepared by addition of appropriate concentrations of ligand to the eight total drug QCs, resulting in four pairs of active QCs with concentrations of 0.2, 0.6, 2.5 and 16 µg/ml (Figure 3).

The two lowest total QCs (QCs: 1 and 2, 0.4 and 1.0 µg/ml) were intentionally quenched to an active drug concentration of 0.2 µg/ml, which was slightly below the limit of quantification of the hybrid LC–MS/MS method. This generated a pair of active QCs containing a total drug concentration that would be within the quantitative range of the assay, but should result in a below the lower limit of quantification (BLQ) result in case the assay is indeed providing correct active drug concentrations.

The ‘total’ QCs containing 32 and 80 µg/ml were above the range of the LC–MS/MS assay and were only used for the preparation of the 16 µg/ml active QCs with 50 and 20% active drug, respectively.

Special attention was paid to the effect of incubation time on the assay results, since this has been identified as the most critical parameter because the dissociation of the complexes is driven by the dissociation constant [6,15,16]. The published hybrid LC–MS/MS protocols, also for ‘free’ drug quantification, often used overnight incubation for the extraction step [14,23], in contrast to our published active LBA method [25], which was time optimized to be as fast as possible. In this example, even the typically applied 1-h incubation step for LBA procedures resulted in significant dissociation of complexes and thus erroneously high assay results.

Thus, the active QCs were processed with a ‘long’ incubation time (overnight) and a ‘short’ incubation time of 10 min. This enabled us to make a direct comparison of the results and thus to see the effects of the incubation time on the assay accuracy and precision results.

To confirm the correct quantification of the active QCs using the hybrid LC–MS/MS assay, they were also analyzed using the Gyrolab LBA as an orthogonal method. The Gyrolab technology was selected because it offers automated, fast assay performance including a short capturing step.

As shown in Table 2, the applied target-capture hybrid LC–MS/MS procedure performed well, as demonstrated by the accuracy and precision data for the conventional total QCs, which were almost all within the typical acceptance criteria for bioanalytical methods, with just one falling slightly outside the criteria (79% accuracy for the 5 µg/ml total QC with overnight incubation).

No significant difference between the ‘short’ and ‘long’ incubation procedure could be observed for the total QCs in terms of recovery and coefficient of variation, which indicates that the incubation time does not affect the assay performance.

The active QCs, which should fall into the assay range, were analyzed using the Gyros assay (at a higher MRD to adapt to the higher sensitivity of the technology) and the hybrid LC–MS/MS method (for the two sets of active QCs prepared with different incubation times). The identical calibrators were used for both assays.

The Gyros data proved the successful preparation of the active QCs. The active drug concentration, which was achieved by mixing defined amounts of drug and ligand and subsequent calculation using the binding affinity (equilibrium constant $[K_D]$, could be determined with good accuracy [90–103%] and precision [0.3–3.1%]). The fact that this is possible even for QCs with identical active drug concentrations but derived from different total drug concentrations by defined quenching of 50 and 80% using ligand at all concentration levels, provides confidence in the power and flexibility of the ‘free (active) analyte’ QC concept.

The LC–MS/MS analysis of the active QC panel prepared using the short (10 min) and long (overnight) incubation periods provided discrepant results, in contrast to the results observed for the total QCs. The results of the active QCs that fell within the assay range prepared using the short incubation time were within the acceptance range of $\pm 20\%$ of the expected (Table 1; accuracy: 80–120%, precision: 2–16%). As expected, the low concentration pair of active QCs, which contained detectable total drug concentrations of 0.4 and 1.0 $\mu\text{g/ml}$ reduced by addition of appropriate amounts of ligand to a below-assay-range active drug concentration of 0.2 $\mu\text{g/ml}$, were both correctly detected as BLQ.

In contrast, analysis of the identical active QCs prepared with a long incubation time, as described in the literature [14,23], resulted in accuracies that were significantly out of the acceptance range (154–277%). The low active QC of 0.2 $\mu\text{g/ml}$, which should be BLQ, provided signals that corresponded to accuracies of 251% (50% active drug) and 354% (20% active drug). These data showed the remarkable influence of the incubation time on the accuracy of the method, which results from the significant dissociation of the drug–ligand complexes upon overnight incubation.

The significance of appropriate active drug assay development, particularly for hybrid LC–MS/MS procedures, which typically employ rather long incubation times compared with those used for LBAs, can be very nicely illustrated by taking a closer look at one of the total/active QC pairs. Figure 4 shows the results of the hybrid LC–MS/MS analysis of the active QCs prepared from the total QCs containing 1.2 and 3.0 $\mu\text{g/ml}$, which were reduced by 50 or 80%, respectively, by addition of the ligand to give a pair of active drug QCs with concentrations of 0.6 $\mu\text{g/ml}$. The solid lines indicate the target concentrations (1.2 and 3.0 $\mu\text{g/ml}$ for the total QCs; 0.6 $\mu\text{g/ml}$ for both active QCs) and the dotted lines indicate the acceptance range of $\pm 20\%$. The results from the use of the total QCs, as requested by the guidelines and white papers [8,17–20], indicate that the assay performs well with no difference between the two incubation protocols, as shown by the accuracy data falling within the acceptance range. However, these results could wrongly be interpreted as showing that the assay is appropriate for accurate active drug quantification since the method uses a target-binding step as a surrogate to monitor drug activity and the QC performance is within the acceptable range. However, analysis of the samples using a long incubation protocol, as described in the literature [14,23], could provide misleading data since the complexes are dissociated and thus, higher putative active drug concentrations were determined (1.07 and 1.66 $\mu\text{g/ml}$ instead of 0.6 $\mu\text{g/ml}$), incorrectly indicating much higher active drug exposure than that actually present in the sample. This could lead to misinterpretation of the exposure data and incorrect decision-making, if the exposure information is crucial such as in preclinical and clinical studies.

In the case of the 1.2 $\mu\text{g/ml}$ total and 0.6 $\mu\text{g/ml}$ free QC (left example in Figure 4), the method-induced equilibrium shift was so significant that the obtained result of 1.07 $\mu\text{g/ml}$ falls into the acceptance range for correct total drug quantification. Consequently, the supposed active drug assay actually provides ‘total’ drug data.

In contrast, application of the ‘short’ sample processing and extraction, according to the theoretical considerations [6,15,16], also results in correct quantification of free drug concentrations within the acceptance range. This proves that hybrid LC–MS/MS procedures are in principle, suitable for active drug quantification, but require appropriate assay development and characterization. For routine analysis, especially with large sample batch sizes, the complete assay procedure could be monitored, when the QC samples are placed accordingly.

Conclusion

The evaluation described in this work indicates that the use of the ‘free/active analyte QC concept’ [16] enables appropriate method development and characterization, and is suitable for hybrid LC–MS/MS method validation, as described for the validation of active drug LBA assays [15]. Furthermore, using the example of incubation time,

Figure 4. Impact of incubation time on the active assay.

Total and active QC samples were compared in terms of the applied overnight or 10 min incubation. Method-induced equilibrium shift suggests for the active QC, that mAb <X> dissociates from the ligands and becomes total QCs with time.

conc.: Concentration; mAb: Monoclonal antibody; ON: Overnight; QC: Quality control.

we have demonstrated the importance of carefully assessing the impact of all variables on the quantification of free/active drug.

Specific assay development is critical owing to the different binding kinetics between different drugs and binding partners, which requires analyte-specific optimization of the ligand-binding step. This, in turn, enables bioanalytical scientists to provide clearly defined bioanalytical data [1] to the project teams, which is important for clear communication between all stakeholders and thus enables clear decision-making.

Future perspective

The technical proof of an accurate active drug quantification using hybrid LC–MS/MS methods reported herein, illustrates the critical role of the ligand-binding extraction step, which requires careful development. Drug-specific antibodies, for example, anti-idiotypic antibodies, to capture a therapeutic monoclonal antibody, are often used in hybrid LC–MS/MS protocols [14] as a surrogate for the target, which further increases the need for careful assessment of the potential impact of the extraction step on the correctness of the results [7]. In particular, antibodies with displacement binding properties should be avoided. The application of the ‘free/active analyte QC approach’ allows the identification of whether the captured reagent impacts the drug–ligand equilibrium and enables appropriate immunoaffinity extraction development. Consequently, it broadens the bioanalyst’s toolbox since hybrid LC–MS/MS might become a viable alternative to LBAs. However, the most critical limitation is still the limited sensitivity of the MS detection. Despite the huge progress in improving the sensitivity of MS instruments in recent years, the sensitivity limit of approximately 100 ng/ml total drug [26] is still not sufficient for appropriate project support in many cases. Active drug quantification requires higher assay sensitivity *per se* since only a subfraction of the total drug concentration needs to be determined. Depending on the respective concentrations of the soluble binding partner, a differentiation between active and total drug might only be relevant at low concentrations, in the

ng/ml range or below. Since the potency of modern therapeutic proteins is increasing, resulting in lower doses and consequently, drug concentrations, and since clinical studies are increasingly being conducted using the minimal anticipated biological effect level or minimum effective dose [27,28], the need for highly sensitive bioanalytical methods for accurate determination of active drug exposure is increasing. Currently, due to their higher sensitivity and higher throughput capabilities, LBA technologies are advantageous over LC–MS methods and remain the first choice for determination of active drug exposure. Further improvements, particularly to the sensitivity of MS instruments, are required to cope with the decreasing drug doses and serum concentrations encountered in preclinical and clinical studies.

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Summary points

Background

- Accurately determining the free/active forms of novel drugs can be crucial during all stages of development.
- Depending on the assay format used, the concentration of either the total drug, the target-binding competent drug or the active drug could be quantified.
- Accurate quantification of the absolute active drug concentration within a mixture of drug–ligand complexes, without perturbing the equilibrium, is challenging.
- Ligand-binding assays are typically used, ideally using the target as capture reagent.
- Bottom-up LC–MS/MS analysis has been reported for quantification of 'total' drug.
- A drug-specific capturing step (e.g. using an anti-idiotypic antibody) in a hybrid LC–MS/MS method has been claimed to enable free/active drug quantification.
- Appropriate method development and assessment of critical factors is essential; here it is shown that, this can be facilitated by the 'free analyte QC concept'.

Experimental

- A hybrid method involving an anti-idiotypic capture antibody and downstream LC–MS/MS analysis was used to determine free/active therapeutic monoclonal antibodies within spiked serum samples.
- The Gyrolab™ technology was applied as an orthogonal ligand-binding assay technology to confirm LC–MS/MS results.
- Total quality controls (QCs) were prepared at 0.2, 0.6, 2.5, 16, 32 and 80 µg/ml.
- The total QCs were subsequently quenched by ligand to give four pairs of free/active QCs containing 50 or 20% free drug at concentrations of 0.2, 0.6, 2.5 and 16 µg/ml.
- The effect of the incubation time (10 min vs overnight) on the free/active drug quantification by the hybrid LC–MS/MS method was assessed using the 'free analyte QC concept'.

Results & discussion

- The target-capture hybrid LC–MS/MS procedure performed well, as demonstrated by the accuracy and precision data for the conventional total QCs.
- No significant difference was observed between the 'short' and 'long' incubation procedures for the total QCs.
- The active QCs were analyzed using the Gyros assay and the hybrid LC–MS/MS method using identical calibrators. The Gyros data proved the successful preparation of the active QCs. For LC–MS/MS, the short incubation time gave active QC results within the expected acceptance range, but the long incubation time resulted in significant overestimation of the free/active QCs to within the expected range of the total QCs.

Conclusion

- Hybrid LC–MS/MS procedures are in principle suitable for active drug quantification, but require appropriate assay development and characterization, which is facilitated by the use of the 'free/active analyte QC concept'.
- The importance of carefully assessing the impact of all variables on the correct quantification of free/active drug is highlighted through our example using different incubation times.

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