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Reduction of matrix interferences by the combination of chaotropic salt and DMSO in a broadly applicable target-based ELISA for pharmacokinetic studies of therapeutic monoclonal antibodies

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ABSTRACT

Use of a synergistic effect of DMSO together with a chaotropic salt (NaSCN or $MgCl_2$) allowed to drastically reduce matrix interferences in an ELISA for therapeutic monoclonal antibodies. Optimum combinations were found to be 0.4 M NaSCN together with 10.0% DMSO, and 1.0 M $MgCl_2$ with 15.0% DMSO. At this optimum combination, quality controls spiked with mAb at 50.0 ng/ml in eighteen individual human sera and plasmas were quantified with an overall accuracy of 102.0%. All of these QCs fulfilled the acceptance criteria of 80.0–120.0% accuracy and precision below 20.0%. The assay was also successfully applied to the quantification of two other mAbs in human serum. Furthermore, the use of the assay was extended to pre-clinical species (cynomolgus monkey and rat serum). Here, the performed validation experiments confirmed the utility of the assay and demonstrated that the assay allowed quantification of mAb from 50.0 ng/ml to 100.0 μg/ml in cynomolgus monkey serum. The method has then been applied to a pharmacokinetic study in cynomolgus monkeys. In summary, this work demonstrates the efficacy of the combination of a chaotropic salt with DMSO to minimize matrix interferences in an ELISA. The robustness thus obtained allowed the successful establishment of a cost effective, target-based ELISA format for use in pharmacokinetic studies, that is easily applicable for the quantification of mAbs in various matrices such as human, cynomolgus monkey or rat serum and plasma.

1. Introduction

The study of antibodies and other proteins, whether for therapeutic use or not, requires accurate and sensitive immunoassays for their quantification within complex biological milieu such as serum or plasma. The quantification of therapeutic monoclonal antibodies (mAbs) can be performed using mass spectrometry methods [1,2] or by application of immunoassays [3]. Until now, immunoassays are the only methods displaying sufficient sensitivity to measure low doses of mAbs in humans and in some monkey and rodent species as needed for pharmacokinetic studies. While lower limits of quantification of mass spectrometry methods are established at around 500.0 ng/ml [1], concentrations as low as 50.0 ng/ml can be quantified using immunoassays [3]. Nevertheless, due to the complexity of biological milieu, that remains the central source of many interferences, the development and validation of accurate and sensitive immunoassays often is a work intensive challenge.

Matrix interferences frequently occur in immunoassays. They are the result of interaction between matrix components, the analyte and/or assay reagents and lead to inaccurate measurement of the analyte. Proteins (albumin, rheumatoid factors, heterophilic antibodies, etc.), lipids, carbohydrates, small molecules or salts constituting biological samples are all components likely to interfere in immunoassays [4]. Especially, antibody interferences have

Abbreviations: Ab, antibody; mAb, monoclonal antibody; AP, alkaline phosphatase; CV, coefficient of variation; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ELISA, enzyme linked immunosorbent assay; EC50, half maximum effective concentration; EDTA, ethylene diamine tetraacetic acid; LLOQ, lower limit of quantification; $MgCl_2$, magnesium chloride; NaSCN, sodium thiocyanate; NHS-PEO_4, N-hydroxysuccinimide-polyethylene oxide; NSB, non-specific binding; PBS, phosphate buffered saline; PK, pharmacokinetic; pNPP, p-NitroPhenyl Phosphate; QC, quality control; RLU, relative luminescence unit; RT, room temperature; TBST, tris buffered saline with Tween 20 0.05% (v/v); ULOQ, upper limit of quantification.

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been well described in immunoassays, for example the effects of heterophilic antibodies [5] autoantibodies [4,6] and rheumatoid factors [4,7]. In the case of the quantification of mAbs, one of the key difficulties is their similarity to endogenous immunoglobulins, which are highly abundant in plasma. The matrix complexity in combination with the high inter-individual variability makes the specific detection of mAbs very complicated.

During assay validation, accuracy of an immunoassay has to be established within specified limits (generally 80.0–120.0% [8]). Matrix interferences result in a deviation of the measured concentration from the true analyte concentration [4], this effect being especially pronounced at low analyte concentrations. Therefore, because of inaccuracies caused by matrix interferences, an immunoassay often can only be validated successfully at a higher lower limit of quantification (LLOQ) than initially expected. In the worst case, the presence of matrix interferences make the validation of an immunoassay impossible.

To reduce matrix interferences, it is common to dilute the samples or extract the analyte before analysis in the immunoassay [9]. However, sample dilution typically results in loss of sensitivity and analyte extraction is not always applicable. Another approach to reduce interferences is to adjust the samples' environment so that protein–protein interactions are modified. In particular, hydrophobicity and salt composition are two parameters which were shown to modify protein conformation [10,11] and protein–protein interactions [12,13]. Chaotropic salts like sodium thiocyanate (NaSCN) or magnesium chloride ($MgCl_2$) are widely used for protein denaturation and the dissociation of complexes [12]. These salts have been used in some immunoassays to reduce non-specific binding (NSB) and to improve assay performances and specificity [14,15]. Organic solvents such as dimethylsulfoxide (DMSO) are also used to modify protein structures and binding properties [16–18]. The ability to modify protein structures, as well as to dissolve hydrophobic components of the matrix such as lipids, may be exploited to reduce matrix interferences.

In the present study, the potential of two chaotropic salts ($MgCl_2$ and NaSCN) and of DMSO has been evaluated to reduce matrix interferences in an enzyme linked immunosorbent assay (ELISA) for the quantification of therapeutic monoclonal antibodies. The assay is based on the specific capture of the mAb present in a biological sample by its target immobilized on the plate surface, and detection with an anti-human IgG labeled to alkaline phosphatase (AP).

Assay development was performed using therapeutic monoclonal antibody mAb1 in human serum as development model. Assay parameters (NSB and sensitivity) were first optimized in the presence of chaotropic salts and DMSO. Matrix interferences, more pronounced at low mAb concentrations, were then evaluated with quality control (QC) samples spiked with mAb1 at 50.0 ng/ml (targeted LLOQ). Matrix interferences were considered as controlled when the accuracy of QC samples could be established between 80.0 and 120.0%. Analytic performance was thereby optimized first at the LLOQ. Subsequently, QCs were evaluated over the whole working range of concentrations to ensure adequate performance of the assay over the complete working range.

Successful application of the assay to additional mAbs (mAb2 and mAb3) as well as to matrix obtained from other species was demonstrated in subsequent experiments. In the presence of a specific mixture of a chaotropic salt and DMSO in sample diluent, the assay was shown to be accurate and sensitive in all species matrices and for all mAbs tested. In addition, a first validation performed in cynomolgus monkey serum demonstrated the robustness of the assay and met the criteria for the quantification of 50.0 ng/ml to 100.0 μg/ml of mAb in samples obtained from this species.

2. Materials and methods

2.1. Reagents

Therapeutic monoclonal antibodies were supplied by Novartis Pharma AG (Basel, Switzerland). Recombinant human targets were either provided by Novartis or obtained from R&D Systems (Minneapolis, MN) and Peprotech (London, UK). Mouse monoclonal anti-human IgG conjugated to alkaline phosphatase was from Sigma–Aldrich Chemical Co. (St. Louis, MO). N-hydroxysuccinimide-polyethylene oxide ($NHS\text{-}PEO_4$)–biotin for protein biotinylation and SeaBlock blocking buffer were purchased from Pierce Biotechnology Inc. (Rockford, IL). ELISA plates were obtained from Nunc (Roskilde, Dk). Alkaline phosphatase substrates (p-NitroPhenyl Phosphate (pNPP) solution and LumiPhosPlus) were respectively from Uptima (Montluçon, France) and Lumigen Inc. (Southfield, MI). NaSCN and $MgCl_2$ were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). DMSO (gas chromatography quality grade) was purchased from Merck (Darmstadt, Germany). Human sera and human plasmas were obtained from the Etablissement Français du sang d'Alsace (Strasbourg, France). They were collected from healthy and untreated individuals. Cynomolgus sera were obtained from Louis Pasteur University (Strasbourg, France) and rat sera from Inotech AG (Dottikon, Switzerland).

Assay buffer was composed of 2.0% (v/v) SeaBlock blocking buffer in tris buffered saline with 0.05% (v/v) Tween 20 (TBST).

2.2. Conjugation of recombinant human targets with biotin

$NHS\text{-}PEO_4$–Biotin was added to recombinant human protein solution (no less than 1.0 mg/ml in phosphate buffered saline (PBS)) at a 10-molar biotin excess. Reagents were allowed to interact for 45 min at room temperature (RT) with shaking. Excess of biotin was removed by extensive dialysis in PBS.

2.3. Monoclonal therapeutic antibodies calibrators and QCs

Calibrators from 6.3 to 6400.0 ng/ml were prepared by successive dilutions of therapeutic mAb stock solution in an individual serum batch (human, cynomolgus or rat). Calibrator 0.0 ng/ml corresponds to neat serum.

To prepare QC samples, therapeutic mAbs were spiked at the targeted concentration (50.0–1600.0 ng/ml) in several individual serum or plasma batches, different from the batch used for calibrators' preparation.

2.4. Effect of chaotropic salts and DMSO on matrix interferences

To evaluate the effect of chaotropic salts and DMSO on matrix interferences, mAb calibrators and QCs prepared as described above were diluted 1:10 in assay buffer supplemented or not with chaotropic salts and/or DMSO.

Tested concentrations for $MgCl_2$ were: 0.0, 0.6, 0.9, 1.5, 2.0 and 2.7 M. NaSCN was tested at 0.0, 0.2, 0.4, 0.8 and 1.1 M.

DMSO was tested at concentrations from 0.0 to 40.0% (v/v) alone or in combination with $MgCl_2$ 1.0 M, $MgCl_2$ 2.0 M, NaSCN 0.4 M and NaSCN 0.8 M.

Indicated concentrations correspond to final concentrations in diluted sample.

2.5. ELISA

96-well flat-bottom plates with covalently coupled streptavidin (Nunc Immobilizer™) were washed three times with 300 μl/well TBST before 100 μl of biotinylated target (3.0 nM in assay buffer)

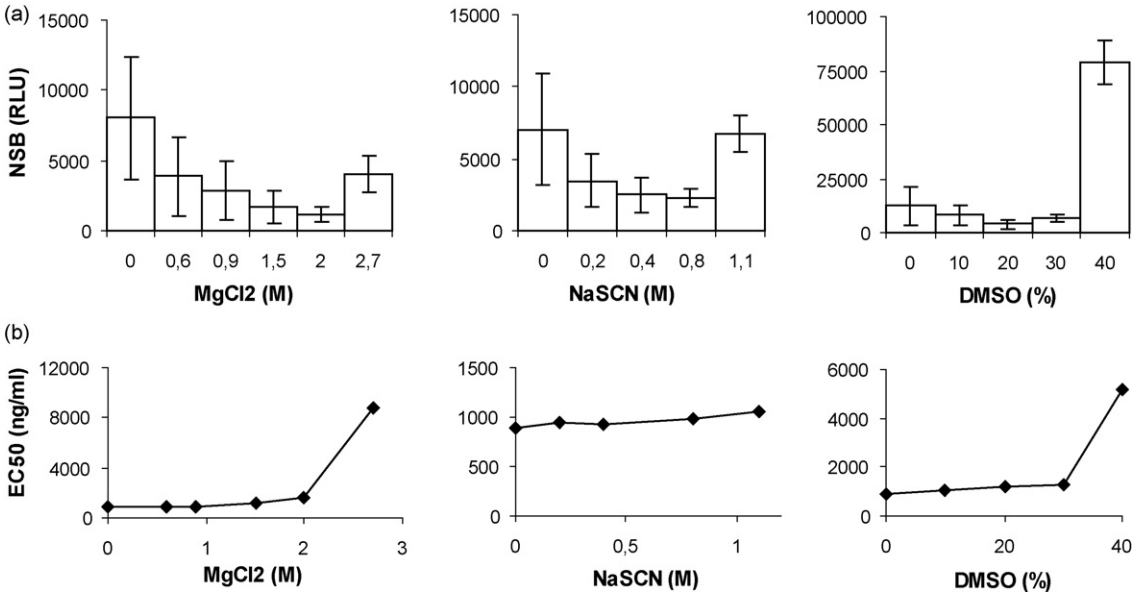

Fig. 1. Effects of $MgCl_2$, NaSCN and DMSO on (a) NSB (expressed in RLU) and (b) EC50. Human sera ($n = 5$) were diluted 10-fold in assay buffer containing increasing concentrations of $MgCl_2$, NaSCN or DMSO. NSB was determined using neat human sera. mAb1 was spiked in an individual serum batch at concentrations varying from 0.0 to 6400.0 ng/ml. Samples were then diluted 10-fold with increasing concentrations of $MgCl_2$, NaSCN or DMSO. EC50 of obtained calibration curves were calculated using a four-parameter regression.

was pipetted into each well. After 1.5-h incubation at RT with 200-rpm shaking, plates were washed three times with 300 μl/well TBST. 10-fold diluted calibrators and QCs were added to predetermined wells at 100 μl/well, in duplicate. Samples were let to incubate on the plate at RT for 2 h under 200-rpm shaking. After washing the plate three times with 300 μl/well of TBST, 100 μl of detection antibody (mouse monoclonal anti-human IgG-AP 1:80,000-diluted in assay buffer) was added to each well followed by incubation at RT for 1 h under shaking (200 rpm). Plates were washed three times with 300 μl/well of TBST, and chemiluminescent or colorimetric AP substrate was added to the plate (100 μl/well). Luminescent or colorimetric reaction was let to proceed in the dark at RT. Luminescence was read after 30 min of enzymatic reaction, with a SpectraMax M5 ELISA plate reader (Molecular Devices, Sunnyvale, CA). Luminescence was expressed in relative luminescence units (RLU), for a signal integrated over 1000 ms. For colorimetric detection, optical density at 405 nm was read after 90 min of color development, with a SpectraMax 340PC ELISA plate reader (Molecular Devices, Sunnyvale, CA). Calibration curves were established using a four-parameter curve fitting model (SoftMax Pro 5.0, Molecular Devices, Sunnyvale, CA). The half maximum effective concentration (EC50) was defined as the concentration of drug antibody required to reach 50% of the signal amplitude between maximum and minimum signals. NSB was defined as the signal obtained with calibrator 0.0 ng/ml. Calibration curves were established for each sample diluent tested. Detected mAb concentration in QC samples was determined using the standard curve established with the corresponding sample diluent.

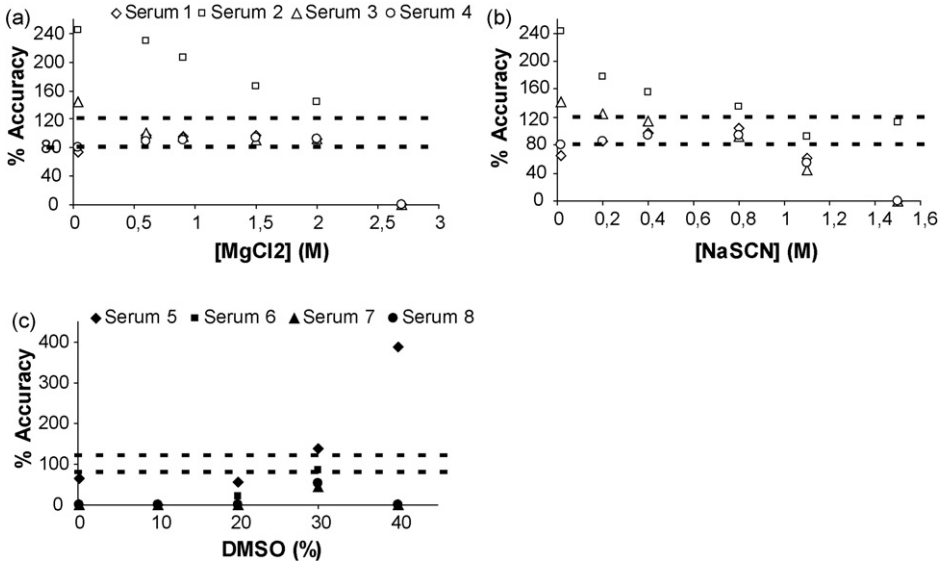

Fig. 2. Accuracy of mAb1 quantification in the presence of increasing concentrations of (a) $MgCl_2$, (b) NaSCN, (c) DMSO. QC samples ($n = 4$) spiked with 50.0 ng/ml of mAb1 were diluted 10-fold in assay buffer containing increasing concentrations of chaotropic salts or DMSO. Dot lines represent acceptance limits for accuracy (80–120%).

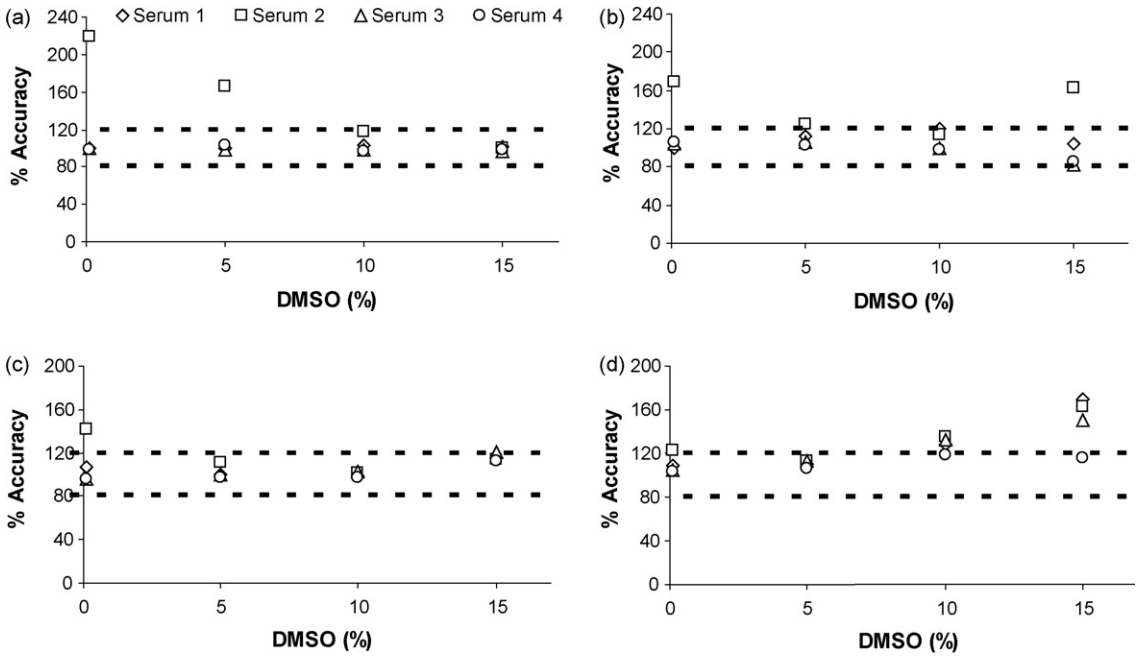

Fig. 3. Accuracy of mAb1 quantification in the presence of increasing amounts of DMSO and (a) $MgCl_2$ 1.0 M, (b) $MgCl_2$ 2.0 M, (c) NaSCN 0.4 M, (d) NaSCN 0.8 M. QC samples ($n = 4$) spiked with 50.0 ng/ml of mAb1 were diluted 10-fold with increasing concentrations of DMSO and a fixed concentration of chaotropic salt. Dot lines represent acceptance limits for accuracy (80–120%).

3. Results

3.1. Effect of chaotropic salts and DMSO on assay parameters

NSB was measured in five different human serum batches after dilution in assay buffer containing increasing amounts of $MgCl_2$, NaSCN and DMSO. NSB could be drastically reduced and homogenized between different serum batches in the presence of $MgCl_2$, NaSCN or DMSO in sample diluent (Fig. 1a). Optimum concentrations to decrease non-specific signal without loss of sensitivity were found to be 2.0 M $MgCl_2$, 0.8 M NaSCN and 20.0% DMSO. At higher concentrations of the three agents, background signal was increased. Sensitivity was degraded in the presence of more than 2.0 M $MgCl_2$ or 30.0% DMSO (Fig. 1b).

3.2. Reduction of matrix interferences with chaotropic salts and DMSO

Accuracy of mAb quantification was measured using QC samples spiked with mAb1 at 50.0 ng/ml in individual serum batches. Accuracy and precision (CV, %) were calculated according to the following formulas:

$$\%\text{accuracy} = \frac{\text{measured concentration}}{\text{spiked concentration}} \times 100$$

$$\%\text{CV} = \frac{\text{standard deviation}}{\text{mean accuracy}} \times 100$$

We defined accuracy lower than 80.0% or higher than 120.0% as the result of matrix interferences.

As shown in Fig. 2, dilution of calibrators and QC samples in assay buffer containing increasing amounts of a chaotropic salt ($MgCl_2$ or NaSCN) or DMSO allowed to progressively decrease matrix interferences. Out of the four QCs, only one was measured within the defined limits (80.0–120.0% accuracy) in assay buffer without neither chaotropic salt nor DMSO. In the presence of 2.0 M $MgCl_2$ or 0.8 M NaSCN, interferences were reduced so that three out of the four QC samples could be quantified with 80.0–120.0% accuracy. However, higher concentrations of these salts disabled any accurate quantification. These concentrations corresponded to those at which NSB increased again. An effect of DMSO could be observed starting from a concentration of 20.0%. In the presence of 30.0% DMSO, mean accuracy was 79.9% but a high variability between individual serum batches could still be observed (CV = 52.9%).

Since none of the three agents tested alone allowed to completely remove matrix interferences, different combinations were tested. Concentrations of chaotropic salts were kept at two levels: maximum effective concentration (2.0 M $MgCl_2$ or 0.8 M NaSCN) and half maximum effective concentration (1.0 M $MgCl_2$ or 0.4 M NaSCN), also corresponding to a maximum signal to noise ratio (data not shown).

Combining $MgCl_2$ with NaSCN did not improve QC accuracy and increased variability between QC samples (data not shown). However, a synergistic effect was observed when DMSO was combined with either $MgCl_2$ or NaSCN, allowing to remove residual matrix interferences. As can be seen in Fig. 3, most effective combinations were found to be 0.4 M NaSCN + 10.0% DMSO (mean accuracy = 99.9 ± 2.6%, $n = 4$) and 1.0 M $MgCl_2$ + 15.0% DMSO (mean accuracy = 99.2 ± 2.2%, $n = 4$).

Eighteen QCs prepared at 50.0 ng/ml in human sera and human plasmas (plasmas collected with either heparin, EDTA or citrate as anticoagulant) were quantified over 3 days with a calibration curve established in an individual human serum batch. Only 22.2% of the QCs diluted in assay buffer without chaotropic salt or DMSO were quantified with 80.0–120.0% accuracy (Table 1). The eighteen QCs could be measured with 80.0–120.0% accuracy in the presence of 0.4 M NaSCN and 10.0% DMSO (Fig. 4a). In the presence of $MgCl_2$ 1.0 M and 15.0% DMSO, QCs prepared in human sera were all quantified within 80.0–120.0% accuracy whereas only 57.1% of the QCs prepared in human plasma samples could be quantified within these limits (Fig. 4b). Accuracy of the assay was also checked over the whole working range of concentrations using six individual batches of human serum and plasma (Table 1).

The combinations 0.4 M NaSCN + 10.0% DMSO and 1.0 M $MgCl_2$ + 15.0% DMSO in sample diluent displayed equivalent efficiency to reduce matrix interferences in serum samples, allowing

Table 1
Accuracy of mAb1 quantification using as sample diluent assay buffer and assay buffer supplemented with 0.4 M NaSCN and 10.0% DMSO. QC samples include matrices such as human serum, human plasma EDTA, human plasma heparin and human plasma citrate. For the quantification of all QCs, calibration curve was prepared in an individual human serum batch.

Nominal mAb concentration	*n*	Assay buffer		Assay buffer + 0.4 M NaSCN + 10.0% DMSO	
		% Accuracy	QCs between 80 and 120% accuracy	% Accuracy	QCs between 80 and 120% accuracy
ULOQ: 1600.0 ng/ml	6	135.6 ± 12.5	0.0%	93.2 ± 4.9	100.0%
800.0 ng/ml	6	137.9 ± 16.6	33.3%	91.8 ± 1.6	100.0%
400.0 ng/ml	6	131.0 ± 24.7	33.3%	95.9 ± 1.4	100.0%
LLOQ: 50.0 ng/ml	18	106.5 ± 149.1	22.2%	102.4 ± 7.1	100.0%

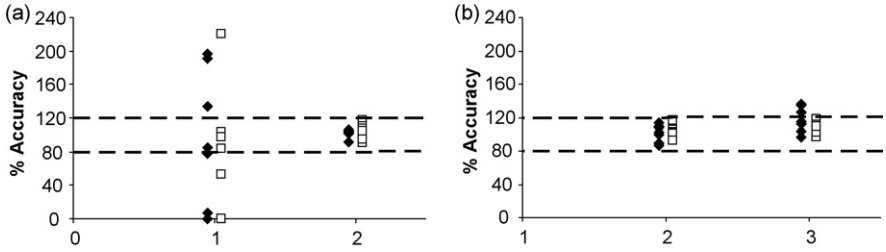

Fig. 4. Accuracy of mAb1 quantification in (1) assay buffer, (2) assay buffer + 0.4 M NaSCN + 10.0% DMSO, and (3) assay buffer + 1.0 M $MgCl_2$ + 15.0% DMSO. Calibration curve was established with mAb1 spiked in an individual serum batch. QCs: human sera (open squares) and human plasmas (full diamonds) were spiked with mAb1 at 50.0 ng/ml and diluted 10-fold in diluents. Dot lines represent acceptance limits for accuracy (80–120%).

Table 2
Accuracy of QC samples spiked with mAb2 and mAb3 at LLOQ and ULOQ in four individual human serum batches. Sample diluent contained 0.4 M NaSCN and 10.0% DMSO. Values are reported as mean ± standard deviation.

	Nominal concentration (ng/ml)	mAb2		mAb3	
		Measured concentration (ng/ml)	Accuracy (%)	Measured concentration (ng/ml)	Accuracy (%)
LLOQ	50.0	49.8 ± 2.5	99.5 ± 5.0	51.9 ± 2.4	103.9 ± 4.7
ULOQ	1600.0	1441.8 ± 2.5	90.1 ± 2.3	1576.6 ± 9	98.5 ± 5.3

to set the LLOQ at 50.0 ng/ml. Besides, plasma samples could be accurately analyzed in the presence of 0.4 M NaSCN + 10.0% DMSO in sample diluent.

3.3. ELISA application to other mAbs and other matrices

3.3.1. Application to other mAbs in human serum

In addition to mAb1, the assay was applied to the quantification of two other monoclonal antibodies: mAb2 and mAb3. To apply the assay to other mAbs, corresponding targets were biotinylated and immobilized on the streptavidin plate using a standardized protocol as described in Section 2. Same protocol as described for mAb1 was applied including the following parameters: sample dilution, sample diluent, incubation time, washing steps, detection system.

For each of the mAbs, all QC samples prepared at 50.0 ng/ml (LLOQ) and 1600.0 ng/ml (ULOQ) in four individual human serum batches could be accurately quantified using a calibration curve established in a fifth individual human serum batch (Table 2).

3.3.2. Application to other species matrices

The assay was applied to the quantification of mAb1 in cynomolgus monkey serum and rat serum samples. mAb1 was spiked in four individual batches of each type of matrix at 50.0 and 1600.0 ng/ml. Samples were diluted 10-fold in 0.4 M NaSCN and 10.0% DMSO and quantified using a calibration curve established in an individual batch of cynomolgus or rat serum. The assay was accurate and sensitive both in cynomolgus serum and rat serum (Table 3).

3.4. Assay validation: case of mAb1 in cynomolgus monkey serum

Validation of the assay was performed according to established guidelines [8]. Acceptance criteria for QC samples were accuracy between 80.0 and 120.0% (75.0 and 125.0% at LLOQ and ULOQ) and precision (CV, %) lower than 20.0% (25.0% at LLOQ and ULOQ). 1.0 M $MgCl_2$ and 15.0% DMSO were used in sample diluent.

3.4.1. Assay selectivity

Accuracy of the method was determined with QCs prepared in fourteen individual cynomolgus monkey serum batches, with mAb1 spiked at 50.0 ng/ml (targeted LLOQ) and 1000.0 ng/ml (targeted ULOQ).

All the QCs could be analyzed with accuracy between 75.0 and 125.0% and precision better than 25.0%, with an average accuracy of 104.3% at LLOQ and 94.4% at ULOQ (Table 4).

Table 3
Accuracy of QC samples in cynomolgus and rat serum spiked with mAb1 at LLOQ and ULOQ in four individual serum batches. Sample diluent contained 0.4 M NaSCN and 10.0% DMSO. Values are reported as mean ± standard deviation.

	Nominal concentration (ng/ml)	Cynomolgus serum		Rat serum	
		Measured concentration (ng/ml)	Accuracy (%)	Measured concentration (ng/ml)	Accuracy (%)
LLOQ	50.0	48.3 ± 2.3	96.6 ± 4.7	53.2 ± 1.4	106.4 ± 2.7
ULOQ	1600.0	1513.8 ± 86.7	94.6 ± 5.4	1627.5 ± 107.5	101.7 ± 6.7

Table 4
Accuracy of mAb1 quantification at LLOQ and ULOQ in individual cynomolgus monkey serum batches.

mAb1 concentration	*n*	Mean accuracy ± standard deviation
50.0 ng/ml (LLOQ)	14	(104.3 ± 7.6)%
1000.0 ng/ml (ULOQ)	14	(94.4 ± 9.3)%

3.4.2. Intra- and inter-run precision and accuracy

To determine intra- and inter-run precision and overall accuracy, QC samples were prepared by spiking mAb1 at four levels within the working range of concentrations (50.0–100.0–400.0 and 1000.0 ng/ml) in neat cynomolgus monkey serum. At each level, three sets of QCs were analyzed with 1:10 dilution in assay buffer containing 1.0 M $MgCl_2$ and 15.0% DMSO over 6 days, for a total of eighteen determinations per concentration. Intra- and inter-run precision (CV, %) were both within 2.0–8.0%; overall assay precision did not exceed 9.0% (Table 5).

3.4.3. Linearity of dilution

Samples from pharmacokinetic studies might contain high concentrations of mAb and need to be diluted beyond the minimum assay dilution of 1:10 before analysis. To evaluate if a concentration of mAb1 can be accurately determined after dilution, mAb1 was spiked into an individual batch of cynomolgus monkey serum at concentrations of 2.5–100.0 μg/ml. Samples were then diluted in sample diluent to reach a concentration of 250.0 ng/ml. 1:10 dilution of obtained samples was afterwards applied to follow assay procedure as for any other sample.

Pre-diluted samples could be quantified with 91.1–114.6% accuracy suggesting a minimum effect of dilution in the assay as the samples were diluted within the tested range (Table 6). As a consequence, the assay was shown to be accurate for the quantification of mAb1 within the range of 50.0 ng/ml to 100.0 μg/ml.

3.5. Pharmacokinetic study of mAb1 following an intravenous dose in cynomolgus monkey

Serum concentration–time profiles of mAb1 in cynomolgus monkeys following slow intravenous bolus injection at 10.0 mg/kg were presented in Fig. 5. Corresponding pharmacokinetic parameters were listed in Table 7. All study samples until last time point could be quantified, with a lowest concentration measured at 661.0 ng/ml. Similar serum concentration–time profiles could be obtained between the three animals; they were comparable to typical profiles observed with IgGs in this species. No sudden decrease of serum concentrations could be observed, suggesting that no interference from for instance anti-mAb1 antibodies or mAb1 target occurred during sample analysis.

4. Discussion

The combination of a chaotropic salt ($MgCl_2$ or NaSCN) with DMSO in sample diluent allowed to drastically reduce matrix interferences observed in our assay. These results allowed to build a sensitive, accurate and broadly applicable ELISA for the quantification of mAbs in complex biological matrices.

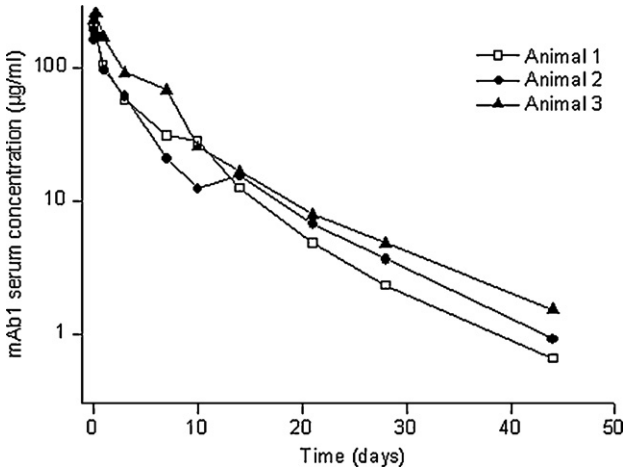

Fig. 5. Serum concentration–time profiles of mAb1 in cynomolgus monkeys after intravenous bolus injection at 10.0 mg/kg. Symbols represent mean measured concentrations. % CV were lower than 3.0% for all determinations.

Table 6
Accuracy of three QC samples spiked with mAb1 at high concentrations and pre-diluted in sample diluent to reach working range of concentrations.

Spiked concentration (ng/ml)	Pre-dilution	Target concentration (ng/ml)	Accuracy (%)
2,500	1:10	250.0	114.6
5,000	1:20	250.0	113.1
50,000	1:200	250.0	91.1
100,000	1:400	250.0	110.9

Table 7
Pharmacokinetic parameters of mAb1 in cynomolgus monkeys after intravenous bolus injection at 10.0 mg/kg.

Time	Animal 1	Animal 2	Animal 3	Mean	% CV
t_{max} (h)	0.170	0.170	2.00	0.780	135.5
C_{max} (μg/ml)	204	193	258	218	15.9
AUC (0–168 h) (h.μg/ml)	11,900	11,300	19,400	14,200	31.8
AUC (0–1056 h) (h.μg/ml)	18,600	17,500	29,100	21,700	29.5
$t_{1/2}$ (h)	174	180	214	189	11.4

To reduce matrix interferences, interactions between the mAb, serum components and assay reagents have to be modified so that only highly specific interactions are allowed to occur. This is what was achieved in the presence of a chaotropic salt and DMSO in sample diluent.

Chaotropic salts are agents with the ability to influence the organization of water molecules in a sample via an effect on hydrogen bonds, leading to modifications in proteins structure and in the way they interact with their environment [12]. The ability of chaotropic agents to dissociate protein complexes [12] had already been exploited in immunoassays to reduce NSB [19] and to increase specificity [15,20]. In our assay, $MgCl_2$ and NaSCN were both able to decrease NSB and matrix interferences. The reduction of NSB can be explained by the property of chaotropic salts to inhibit, at low concentrations, the formation of non-specific interactions (weak

Table 5
Inter-run and intra-run accuracy and precision (CV, %) of mAb1 quantification in cynomolgus monkey serum.

mAb1 concentration	*n*	Overall accuracy (%)	Overall precision (%)	Inter-run precision (%)	Intra-run precision (%)
50.0 ng/ml (LLOQ)	18	107.6	6.8	3.0	5.9
100.0 ng/ml	18	106.0	6.5	3.5	4.3
400.0 ng/ml	18	105.3	8.9	3.4	6.2
1000.0 ng/ml (ULOQ)	18	97.4	8.2	2.0	7.5

interactions) [15,19]. The stringency increases with the amount of chaotropic salt so that only more specific interactions (stronger interactions) are allowed to occur at higher chaotropic salt concentrations. However, the use of a chaotropic salt to remove interfering interactions is limited to the concentration at which the specific mAb–target interaction is inhibited. In our assay, this limit was identified to be at around 2.0 M $MgCl_2$ or 0.8 M NaSCN, concentrations at which EC50 starts to increase. It was also observed that too high concentrations of a chaotropic salt not only inhibited the mAb–target interaction but also led to an increase of non-specific signal, maybe due to the formation of aggregates between serum components and assay reagents.

Since only a limited concentration of chaotropic salt can be used, the complete removal of matrix interferences can only be achieved with the addition of a second agent. As well as chaotropic agents, organic solvents can modify a wide range of physical properties of proteins in solution, like for instance protein conformation [11,21] and interaction [21]. As a result of conformation modification, the binding affinity of monoclonal antibodies to their target can be strongly increased in the presence of organic solvents [16,17]. Melnikova demonstrated that this effect was solvent and antibody specific [16]. Some studies also demonstrated that the specificity of an antibody could be improved in organic media [22]. Likewise, the property of DMSO to modify macromolecular structures and to disrupt hydrogen bonds is exploited in PCR to increase the specificity of DNA strand interactions [23].

The ability of DMSO to reduce matrix interferences was observed at concentrations starting from 20.0%, but high variability between serum batches remained. 40.0% DMSO strongly reduced the binding of the mAb to its immobilized target. The effects of DMSO reported in the previously mentioned studies were observed in buffer solutions and may not reflect the situation in a complex matrix containing high amounts of various proteins. Nevertheless, the reduction of matrix interferences correlated well with the increase of specificity observed by Russell et al. [22].

Whereas neither chaotropic salts nor DMSO alone were able to completely inhibit non-specific interactions, the combination of the two agents was complementary and removed residual interferences. Thus, when 10.0% DMSO alone showed no effect, a synergistic action with a chaotropic salt (0.4 M NaSCN or 1.0 M $MgCl_2$) was observed so that mAb could be quantified without interference in every matrix batch tested. As described by Mande for lysozyme, DMSO and guanidium chloride (a chaotropic salt) can interact at different sites of proteins [24]. It is therefore tempting to interpret the synergistic effect we observed by the action of DMSO and NaSCN or $MgCl_2$ on different types of proteins or at different sites of the same protein.

In addition to DMSO, we have tested two other organic solvents: methanol and dimethylformamide (DMF). DMF and DMSO are both aprotic polar solvents, whereas methanol is a protic polar solvent. If methanol showed poor efficacy in reducing interferences, DMF displayed strong capacities to reduce NSB and interferences already at a concentration of 5.0%. However, DMF alone was not able to remove all interferences. Besides, a synergistic action could be observed with $MgCl_2$ in human serum but not with NaSCN (data not shown). These results suggested that, in complex matrices such as serum, synergistic effects obtained with an organic solvent are chaotropic agent specific. Besides, reduction of interferences cannot be obtained with any organic solvent and further investigations will be needed to determine if this effect is specific to polar aprotic solvents.

The drastic reduction of matrix interferences obtained with the combination of a chaotropic salt with DMSO allowed to develop a sensitive and broadly applicable ELISA format for the quantification of mAbs during pharmacokinetic studies. The quality of all assay reagents (type of ELISA plates, biotinylated recombinant target, detection antibody) appeared to be very important to minimize cross-reactions. However, even after optimization of assay reagents, strong interferences due to matrix components remained. Successful reduction of matrix interferences could be achieved only by diluting samples in a buffer containing a combination of DMSO and NaSCN or $MgCl_2$.

We showed that the assay was applicable for mAbs in various biological matrices, such as serum or plasma of human, cynomolgus or rat origin. Validation parameters demonstrated that the ELISA was robust, reproducible, accurate and sensitive, with a range of quantification of 50.0 ng/ml to 100.0 μg/ml. Application of the assay to a pharmacokinetic study in cynomolgus monkey gave consistent results, in agreement with expected values.

If matrix interferences occur during the development of an immunoassay, the time needed for the successful development and validation can be dramatically increased. In some cases, matrix interferences make a successful assay validation impossible. The ELISA assay format described herein can be quickly and easily applied to the quantification of other mAbs. According to our experience, excellent results in terms of sensitivity and accuracy can be expected already at the first test run. Moreover, as this ELISA utilizes a pre-determined assay format together with standardized commercially available reagents, development and validation timelines will be shortened generally, even without the occurrence of matrix interferences. In consequence, the broadly applicable assay technology we describe here has the potential to decrease the overall time period spent for example for the development, validation and use of pharmacokinetic ELISA assays by as much as 60.0–70.0%.

The lower limit of quantification of an assay not only depends on the sensitivity but also on the presence of matrix interferences. The results obtained in the present study allowed to reach a LLOQ of 50.0 ng/ml by diluting samples with DMSO and NaSCN or $MgCl_2$. For assays that would require quantification at even lower concentrations, a better understanding of the mechanism through which the synergy of chaotropic salts and organic solvents allows to inhibit non-specific interactions could help to define a more powerful chaotropic salt/organic solvent combination. Additionally, the concentrations of chaotropic salts and organic solvent could be adapted for the quantification of biomarkers or other molecules in indirect ELISAs as well as in other enzyme immunoassay formats such as sandwich ELISAs, microsphere assays or competitive assays.

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