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RESEARCH ARTICLE

Detection of cynomolgus monkey anti-protein XYZ antibody using immunocapture-LC/MS

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Although enzyme-linked immunosorbent assays and electrochemiluminescence (ECL) immunoassays are the most widely used platform for ADA detection, they may be compromised by drug interference. We describe here an alternate, free of drug interference, immunocapture-LC/MS methodology for detecting anti-protein XYZ antibody in cynomolgus monkey plasma. In our approach, ADA-protein XYZ complexes are captured by a mouse monoclonal anti-drug antibody on streptavidin magnetic beads and separated from monkey plasma by a magnet. After elution, ADA are digested with trypsin and detected by LC/MS using a surrogate peptide common to monkey IgG subclasses 1-4. The immunocapture-LC/MS assay was applied to support a toxicology study and results were in close agreement with those from a modified ECL immunoassay. To our knowledge, this is the first application of using LC/MS for monkey ADA detection.

Keywords: anti-drug-antibody, drug tolerance, immunoassay, immunocature-LC/MS, universal peptide.

Introduction

Therapeutic proteins and peptides have the potential to elicit immune responses [1,2], resulting in anti-drug antibodies (ADA) that can pose problems for both patient safety and drug efficacy. ADA can affect drug efficacy, biodistribution and drug clearance, and complicate interpretation of toxicity, pharmacokinetic (PK) and pharmacodynamic (PD) data [3-5]. During drug development, immunogenicity is examined using a risk-based approach along with specific strategies for developing "fit-for-purpose" bioanalytical methods [6]. Enzyme-linked immunosorbent assays (ELISA) and electrochemiluminescence (ECL) immunoassays [7] are among the most widely used platform for ADA detection. Other platforms that have been used include surface plasmon resonance and bio-layer interferometry which may be more suited for detection of low affinity ADA [8]. Typically, detection of ADA is followed by assessments of the magnitude (titer) of the ADA response and the in vitro neutralizing ability of ADA, especially in late-stage clinical studies. Additional characterization of ADA such as immunoglobulin subclass or isotype determinations, domain-mapping, relative binding affinity, cross-reactivity with endogenous proteins, or complement activating ability of the ADA may be driven by product-specific, indication-specific, or risk assessment-based objectives [9-11].

In recent years, LC/MS has emerged as a promising platform for quantitation of biotherapeutics and protein biomarkers in biological matrices [12-14]. The vast majority of LC/MS-based protein quantifications are performed at peptide levels, mainly due to consideration of assay sensitivity [15]. A typical procedure for LC/MS-based quantification includes enzyme digestion and quantifica-

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tion of the target proteins based on selected surrogate peptides derived from the target [16,17].

With traditional ADA immunoassay, only drug-free or partially drug-free ADA can be detected, and the presence of high drug levels in study samples can interfere with the detection of ADA, especially in bridging assay formats. Drug tolerance which is generally defined as the maximal amount of free drug in the samples that still allows detection of ADA at an acceptable sensitivity, may be improved using various approaches including the use of excess spiked drug to create and detect drug-ADA complexes rather than free ADA [18-21]. LC/MS has been used to assess ADA in the presence of excess protein therapeutic in support of clinical programs addressing the safety and tolerability of human growth hormone analogues [21]. This methodology overcame drug interference by completely saturating available ADA binding sites with the addition of excess therapeutic. Drug-ADA complexes were then isolated using protein G immobilized on magnetic beads, followed by elution and digestion. Resultant peptide from the target therapeutic protein was quantified by LC coupled with matrix-assisted laser desorption MS and the results were correlated to the binding capacity of total ADA.

Recently, we reported an immunocapture-LC/MS methodology for simultaneous ADA isotyping and semi-quantitation in human plasma [22]. Briefly, ADA or ADA-drug complex was captured by biotinylated drug or anti-drug Ab on streptavidin magnetic beads and separated from human plasma by a magnet. ADA was then released from the beads and subjected to trypsin digestion followed by LC/MS detection of specific universal peptides for each ADA isotype/subclass. Proof-of-concept was demonstrated by detecting pre-existing ADA in human plasma.

We describe here an immunocapture- LC/MS assay for the detection of ADA in cynomolgus monkey plasma in support of a toxicology study. In many of the cynomolgus monkey plasma samples, drug levels were found to exceed the drug tolerance limit of the initial ECL assay. The LC/MS assay and a new ECL assay were developed in parallel to overcome the drug interference issue and results from the two assays were compared. Similar to the human ADA assay [22], a monkey-specific universal peptide was identified and used as the surrogate for the detection of monkey ADA Ig1-4. To our knowledge, this is the first case study using immunocapture-LC/MS for direct ADA detection in support of clinical or nonclinical studies.

Materials and Methods

Protein XYZ is a proprietary experimental biotherapeutic of Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT) and was produced in-house. Protein XYZ was a recombinant protein with a MW of ~39 KDa, consisted of 3 subunits and contained no human Fc. A mouse anti-idiotype monoclonal antibody (mAb) against Protein XYZ was supplied in-house. Monkey IgG1 reference material was purchased from Sigma Aldrich (St. Louis, MO). Surrogate peptide VVSVLTVTHQDWLNGK was synthesized at Genscript (Piscataway, NJ). Streptavidin magnetic beads (1μm diameter.), TPCK trypsin and EZ-Link Sulfo-NHS-LC Biotinylation Kits were obtained from Thermo Scientific (Rockford, IL). Rapigest SF Surfactant was purchased from Waters (Milford, MA). Cynomolgus monkey and dog plasma were purchased from Bioreclamation (Westbury, NY). All other lab chemicals, reagents and buffer solutions were obtained from Sigma Aldrich, Thermo Scientific or Invitrogen (Grand Island, NY).

Monkey plasma samples

The monkey ADA plasma samples were obtained from a toxicology and toxicokinetic (TK) study with weekly intravenous (IV) dosing of Protein XYZ to cynomolgus monkeys for 6 weeks followed by a 13-week recovery period. Monkeys were divided into 4 dose groups: 0 (control), 5, 50 and 100 mg/kg (3-5 monkeys/sex/dose group). Plasma (K_3EDTA) were collected at pre-dose in weeks 1, 3 and 6, and 21, 42, 63 and 84 days after the last dose. The total drug concentrations in the ADA samples of drug-treated monkeys ranged from 0.229 to 1530 μg/mL as determined by a validated sandwich ELISA assay. Drug levels in many of the 100 mg/kg dose group samples were >1000 μg/mL and exceeded the drug tolerance limit of the initial ECL assay.

Biotinylation

Biotinylation of the mouse anti-idiotype monoclonal antibody (mAb) was performed using an EZ-Link SulfoNHS-LC Biotinylation Kit following the vendor instructions. Typical biotin incorporation was determined to be approximately 7 biotins per mouse mAb molecule.

Incubation

For monkey plasma samples with Protein XYZ concentrations of <400 μg/mL, 2 μL of a 10.32 mg/mL drug solution was spiked into 48 μL of the monkey plasma sample to bring the drug concentration up to at least 400 μg/mL. The samples were then incubated at 37°C for 1 hr and stored at -80°C overnight prior to analysis. The samples were then diluted 10-fold with dog plasma prior to immunocapture.

Immunocapture
An aliquot of 10 µL of diluted plasma sample, 100 µL of 0.1 mg/mL biotinylated mouse mAb and 390 µL of Tris-buffered saline with 0.1% Tween-20 (TBS-T) were added to a 96-deepwell polypropylene plate. The plate was then incubated at room temperature for 2 hrs. A 50 µL aliquot of freshly prepared 5 mg/mL magnetic beads was added to each sample and the plate was gently mixed for 1 hr at room temperature. The beads were separated, washed three times with 300 µL of TBS-T and once with 300 µL of water, and then eluted with 150 µL of 0.1 M glycine (pH 2.0) on a Kingfisher Flex magnetic bead handler. The eluent was immediately neutralized with 45 µL of 1 M Tris-HCl (pH 8.0).

Trypsin digestion
To the immunocapture eluent, 5 µL of 0.1% Rapigest in 100 mM ammonium bicarbonate were added and the plate was gently mixed for 5 minutes. Five µL of 50 mM TCEP in 100 mM ammonium bicarbonate were added and followed by incubation at room temperature for 20 min. After adding 5 µL of 50 mM iodoacetamide in 100 mM ammonium bicarbonate, the plate was gently shaken for 20 min while protected from light. A 5 µL aliquot of solution containing trypsin (0.2 mg/mL) and calcium chloride (0.2 M), prepared immediately before use, was added to each sample and the plate was incubated at 37°C overnight with gentle mixing. Digestion was quenched by adding 5 µL of 20% TFA. The samples were mixed for 40 min at 37°C and then centrifuged at 4400 rpm for 10 min prior to LC/MS analysis.

LC/MS analysis
An Eksigent Ekspert MicroLC 200 coupled with an AB Sciex 6500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) was used. Chromatographic separation was performed using an ACQUITY UPLC Peptide BEH C18 column (1 mm x 50 mm, 1.7 µm, 300Å) operated at 60°C. Mobile phases consisted of (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile running at a flow rate of 60 µL/min. The LC gradient was 5% to 50% B over 2.2 minutes. The mass spectrometer was operated in positive electrospray ionization mode. Key instrument parameters were: +5000 V electrospray voltage, 65 nebulizer gas units, 30 axillary gas units, 375°C ion source temperature, 10 collision gas units, and unit resolution on both Q1 and Q3. Single-reaction-monitoring (SRM) with parent-to-product ion transition m/z 599.33 → 1098.53 for VVSVLTVTHQDWLNGK was used for detecting the monkey ADA (IgG1-4). For identifying the surrogate tryptic peptide that is present in monkey IgG1-4, a monkey IgG1 aqueous solution (10 µg/mL) was digested and analyzed by LC/MS using information-dependent acquisition (IDA).

ECL immunoassay
An ECL immunoassay using Protein G sample purification was also developed in parallel for this project. ADA and Drug-ADA complexes were captured using Protein G spin plates in order to remove excess free drug which does not bind to Protein G. After wash steps the captured ADA and/or ADA-drug complexes were subjected to an elution/acid dissociation step. The eluents were then analysed using an ECL drug-bridging assay for ADA detection. A 95th percentile was calculated using the RLU data and used as the cut point. The assay was able to detect 250 ng/mL of a positive mouse ADA control in the presence of up to 1500 µg/mL of Protein XYZ.

Results and discussion
The immunocapture-LC/MS assay was developed to support the toxicology and TK study with weekly intravenous (IV) dosing of Protein XYZ to cynomolgus monkeys. Drug levels in many of the monkey plasma samples exceeded the drug tolerance limit of the initial ECL assay, and thus an immunocapture-LC/MS assay and an improved ECL assay were developed in parallel to overcome the drug interference issue.
In monkey plasma or serum, IgG1-4 are the predominant immunoglobulin isotypes [23], and therefore, only these ADA isotypes were investigated in the present study. The LC/MS ADA detection was based on a surrogate peptide which was from the heavy chain constant regions across all cyno IgG1-IgG4 isotypes [15]. Monkey IgG1-4 ADA in the study samples were expected to have different amino acid sequences in their variable regions but each subclass should have the same target surrogate peptide in their constant regions. Therefore, the surrogate peptide was selected from the constant regions of monkey IgG1-4 in order to detect all IgG1-4 ADA.
The selection of the monkey surrogate peptide involved several steps. First, in silico trypsin digestion of IgG1-4 was performed to generate a list of potential peptide candidates. Peptide size was limited to 6 to 20 amino acid resides which was found to be the right balance between assay specificity/selectivity and sensitivity [22]. The surrogate tryptic peptide must also be a universal peptide and present in all IgG1-4 isotypes, so that it could be used to detect the total of IgG1-4. Trypsin digestion of a 10 µg/mL neat IgG1 phosphate buffer solution was then performed to verify which of those peptides were indeed formed and detected by LC/MS. The most sensitive pep-

tide was identified to be VVSVLTVTHQDWLNGK with SRM parent-to-product ion m/z 599.33 → 1098.53. It should be noted that this peptide could also be produced via trypsin digestion from some cynomolgous endogenous proteins based on BLAST (Basic Local Alignment Search Tool) search. However, these endogenous proteins will not be captured by the mouse mAb. Although they should be removed in the immunocapture steps, any carryover could contribute to the background response. The selectivity and specificity of this assay was assessed with the tryptic digest of blank monkey plasma which were pre-diluted 10x with dog blank plasma and prepared according to the immunocapture procedures. Minimal matrix interference was detected at the SRM channel of m/z 599.33 → 1098.53. Therefore, peptide VVSVLTVTHQDWLNGK was selected as the universal, surrogate peptide for detecting the total of IgG1-4 by LC/MS. Plasma is a very complex matrix that contains several hundreds of thousands of proteins and protein isoforms in a wide concentration range. Upon digestion these can be cleaved into multiple peptides, from which just one or a few peptides have to be quantified. When no protein or peptide purification step is employed, LC/MS sensitivity is significantly compromised due to matrix interference from the peptide-rich digest [24]. For high-sensitivity LC/MS applications, immunopurification is the most effective way to improve assay sensitivity and robustness by isolating target protein from matrix [25,26]. Further immunopurification of surrogate peptide from the digest could be carried out using an anti-peptide Ab [27]. In the present study, we employed an anti-idiotype mouse mAb for immunocapture of Protein XYZ-ADA complexes. The workflow of the immunocapture-LC/MS assay is depicted in **Figure 1**. In this approach, ADA had to be first converted to Protein XYZ-ADA complexes. Biotinylated mouse mAb was then added to capture the drug-ADA complexes along with free drug. In the presence of mAb, the drug-ADA complexes and free drug formed mAb-drug-ADA and mAb-drug complexes, respectively. After adding streptavidin magnetic beads, the complexes were immobilized on the beads and were separated from monkey plasma using a magnet. ADA was then eluted from the beads, digested and assayed by LC/MS. The total drug concentrations in the ADA samples of drug-treated monkeys ranged from 0.229 to 1530 μg/mL. For samples containing < 400 μg/mL drug, additional drug was added in order to ensure that sufficient mAb-drug-ADA complexes were formed and eventually detected by LC/MS. A phosphate buffer solution containing 10.32 mg/mL drug, the most concentrated drug material available, was used for the spiking. In order to maintain matrix integrity, only 2 μl of the spiking solution was added to 48 μL of the ADA plasma sample, which yielded a final total drug concentration of ≥ 413 μg/mL. If we assume that a 2:1 molar ratio of drug: ADA was required for the conversion of ADA to drug-ADA complex, 413 μg/mL drug would convert approximately 800 μg/mL ADA to drug-ADA complex, which was much higher than the LC/MS ADA assay detection limit of ~1 μg/mL (see discussion later on). Our goal was to determine whether the ADA samples were positive or negative, and the spiking experiment should serve the purpose. It has been reported that 100 μg/mL of hGHA was sufficient to convert 100 μg/mL ADA to hGH-ADA complex in human serum [21]. In the immunopurification process, the mouse mAb-drug-ADA complexes were separated from the plasma by a magnet and any endogenous monkey plasma proteins that bound non-specifically on the beads were subsequently washed off. However, it is possible for endogenous monkey IgG1-4 and other plasma proteins to survive the washing process and be carried on to the digestion. These proteins that also give the surrogate peptide after digestion will be detected as a background peak in the LC/MS assay as shown in **Figure 2a**. In the 3.5 min LC run, the surrogate peptide eluted at 1.8 min. The LC/MS response (peak area) of the surrogate pep-

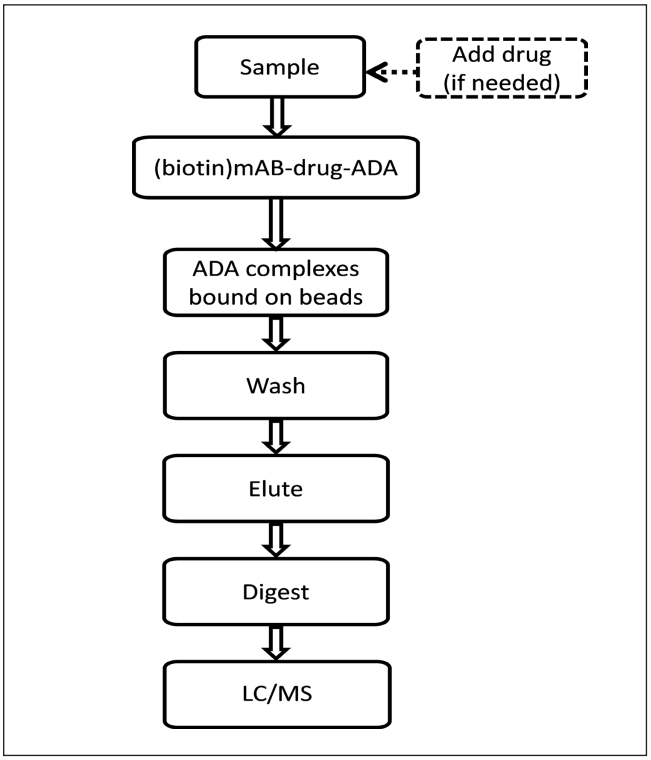

Figure 1. Immunocapture-LC/MS workflow chart.

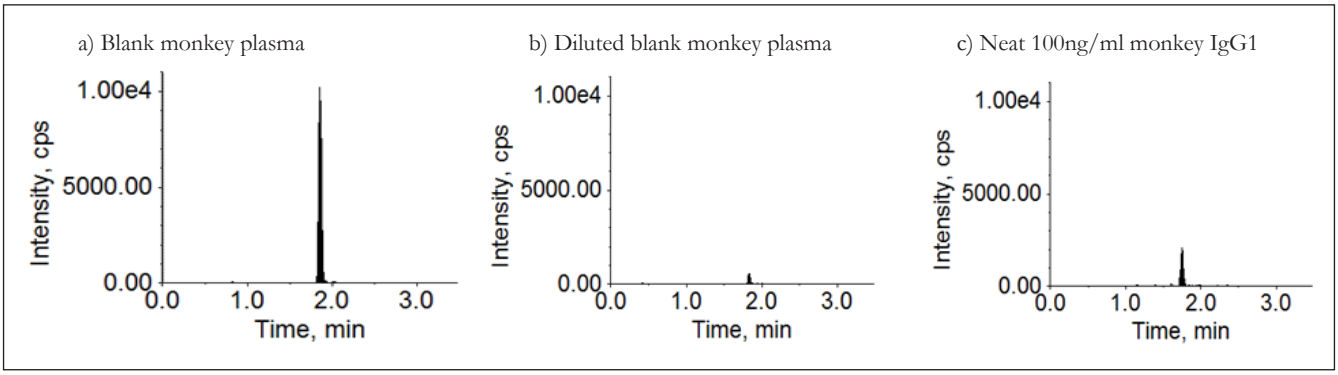

Figure 2. LC/MS chromatograms of surrogate peptide MRM channel m/z 599.33 → 1098.53 from a) blank monkey plasma, b) blank monkey plasma diluted 10-fold with dog plasma prior to immunocapture , and c) 100 ng/mL monkey IgG1 (bottom). The blank plasma samples were prepared following the immunocapture and trypsin digestion procedures. The 100 ng/mL monkey IgG1 sample was a neat trypsin digestion.

tide in the undiluted plasma was as high as 22200, indicating significant non-specific binding of endogenous plasma components. In order to reduce the background response, the monkey samples were pre-diluted 10-fold with blank dog plasma. The LC/MS peak area of the 10-fold diluted blank monkey plasma was 18-fold less than the undiluted sample (**Figure 2b**). To assess the impact of non-specific binding on the assay sensitivity and specificity, a 100 ng/mL monkey IgG1 neat solution digestion was prepared and assayed together with the blank samples (**Figure 2c**). This solution is equivalent to a 1000 ng/mL sample diluted 10-fold. Compared to the 100 ng/mL

Table 1. LC/MS results of validation samples assayed on two different days

Animal #	Timepoint	ECL Readout (cut point = 79)	LC/MS Peak Area			
			Run 1	**Run 2**	**Average**	**% difference***
1	Wk 1, Pre-dose**	66	3770	3840	3805	-1.8
	Wk 1, 5 min***	NA	3950	3450	3700	13.5
	Wk 13**	2527	6550	6310	6430	3.7
2	Wk 1, Pre-dose	61	1780	1470	1625	19.1
	Wk 1, 5 min	NA	1070	1240	1155	-14.7
	Wk 13	2175	1780	2010	1895	-12.1
3	Wk 1, Pre-dose	67	2160	2860	2510	-27.9
	Wk 1, 5 min	NA	1570	1370	1470	13.6
	Wk 22**	2067	23900	20800	22350	13.9
4	Wk 1, Pre-dose	64	1890	2730	2310	-36.4
	Wk 1, 5 min	NA	1730	2030	1880	-16.0
	Wk 22	821	16700	18400	17550	-9.7
5	Wk 1, Pre-dose	274	5300	4290	4795	21.1
	Wk 1, 5 min	NA	4490	3750	4120	18.0
	Wk 22	1061	13700	11700	12700	15.7
6	Wk 1, Pre-dose	64	5030	4210	4620	17.7
	Wk 1, 5 min	NA	4400	4230	4315	3.9
	Wk 22	1576	12200	11200	11700	8.5

*%difference = (Run 1 – Run 2)/Average; **ADA samples; *** TK samples, not assayed by the ECL assay. NA: not available.

IgG1 neat digest, the peak area of the diluted blank plasma was 4-fold less indicating that with the reduced background after dilution, the LC/MS assay was able to detect ADA equivalent to at least 1000 ng/mL in plasma. It is expected that further removal of the non-specific binding would improve the assay sensitivity and specificity. Like any bioanalytical assays, the LC/MS assay needed to be validated prior to sample analysis. Typically, an ECL bridging ADA assay is validated using an ADA positive control (PC), which includes sensitivity, selectivity, precision and drug tolerance in addition to sample stability. The PC may or may not be from same species/strains that the assay is intended for. Mouse PCs are often used as they are readily available in the early drug development. In contrast, a monkey ADA PC is required in order to perform similar validation for the LC/MS monkey ADA assay, since the LC/MS assay is based on the analysis of the unique surrogate peptide that can only be formed from monkey IgG after digestion. For this particular case, the drug development was still in early stage and no monkey ADA PC was available. Therefore, only limited LC/MS assay validation was performed.

Eighteen plasma samples from a different monkey study were selected for the LC/MS assay validation. The sample set was comprised of both ADA negative and positive samples based ECL readout. Sample analyses were conducted on two different days and the results were compared to assess the assay precision. The results expressed in LC/MS peak area are listed in **Table 1**. The differences between the two runs for the 17 out of 18 samples were within ± 30%, indicating good precision.

During ADA immunoassay development and validation, the assay cut point, which is the level of response of the assay at or above which a sample is defined to be positive and below which it is defined to be negative, needs to be determined [6]. Similar to immunoassays, a cut point was established for the immunocapture-LC/MS assay. A total of 84 study samples were used for the cut point determination, which included all the control group samples plus the week 1, predose samples from the 5, 50 and 100 mg/kg drug-treated groups. Also included were some week 1, 5-minute post dose PK samples from the drug-treated groups. All these samples were expected to be ADA negative. The LC/MS ADA responses (peak area), mean and standard deviation (SD), and the calculated cut point are listed in **Table 2**. The LC/MS responses ranged from 196 to 3520, with a mean of 1492 and a standard deviation (SD) of 724. Since these samples were ADA negative, their LC/MS responses were the result of non-specific binding of endogenous plasma components as discussed above. The wide range of LC/MS responses indicated a large variability of non-specific binding among individual monkeys. It was therefore necessary to establish the cut point using these ADA negative samples from the same group of monkeys from which the samples were to be assayed for ADA. The cut point was set to 99% to allow a rate of 1% false positives [28]. Using the standard calculation formula for a 99% cut point: mean+(2.326 x D) [28], the calculated cut point value was 3,176.

Table 3 shows the LC/MS ADA responses of samples from the week 1, week 3, week 6, and recovery phase of the drug treated groups. The LC/MS responses ranged from 917 to 24300. Samples with response less than the cut point of 3,176 were considered to be ADA negative while those of ≥ 3176 were ADA positive. **Figure 3** shows chromatograms of representative ADA negative and positive samples. As expected, all the samples prior to drug treatment (week 1 predose) were ADA negative. On week 3, 4/3/5 out of 6/6/10 monkeys were ADA positive from the 5, 50 and100 mg/kg/week dose groups, respectively. All these ADA positive monkeys remained positive at week 6 and further into the recovery phase (recovery monkeys only). In addition, 1 monkey from the 5 mg/kg/week and 2 from the 100 mg/kg/week dose group became positive at week 6 and remained positive in the recovery phase, while they were ADA negative on week 3. In general, ADA responses (LC/MS peak area) increased from week 3 to week 6 but decreased in the recovery phase. The highest ADA response observed was 23,100 for the males and 24,300 for the females in the week 6, 100 mg/kg/week group, which were approximately 7.5 fold higher than the cut point of 3176. The differences in ADA response between the control and the drug treated group samples as well as among different weeks were clearly illustrated in **Figure 4**. The incidence of ADA was much higher in drug-treated monkey samples than in control group samples. However, it did not appear to be dose dependent. Overall 5 out of 6, 3 out of 6 and 7 out of 10 monkeys from the 5, 50 and 100 mg/kg/week dose groups, respectively, were ADA positive (**Table 3**).

The results from the ECL assay are provided in **Table 3** for comparison. For the 84 samples from the drug treated groups, 24 samples were ADA positive by the ECL assay vs. 25 by the LC/MS assay. Only 1 monkey each from the control, 5 mg/kg/week and 100 mg/kg/week dose group was ADA negative by one assay while positive by the other assay. For all other monkeys, the results were consistent between the two assays, i.e., either ADA negative or positive by both assays.

The merit of using anti-drug mAb as the capture reagent lies in that drug no longer interferes with the assay. This

Table 2. LC/MS responses of samples for cut-point determination

Males			Females		
Subject	Sample time (week)	LC-MS response (peak area)	Subject	Sample time (week)	LC-MS response (peak area)
1302504[a]	1	1960	1302503[a]	1	1270
	3	1890		3	1210
	6	2240		6	1210
1302506[a]	1	1470	1302505[a]	1	1530
	3	2570		3	1140
	6	1540		6	2220
1302508[a]	1	686	1302507[a]	1	930
	3	1050		3	1040
	6	1920		6	912
1302510[a]	1	196	1302509[a]	1	1480
	3	474		3	1470
	6	845		6	1530
	9[b]	1150		9[b]	1740
	12[b]	475		12[b]	2340
	15[b]	661		15[b]	3520
	18[b]	805		18[b]	3320
1302512[a]	1	1630	1302511[a]	1	1350
	3	1490		3	2090
	6	2670		6	1400
	9[b]	2200		9[b]	1810
	12[b]	3130		12[b]	2820
	15[b]	3440		15[b]	3070
	18[b]	2080		18[b]	2210
1302514	1	1140	1302513	1	1530
1302516	1	1220	1302515	1	1400
	1 (5 min)[c]	1150		1 (5 min)[c]	1700
1302518	1	745	1320517	1	1720
	1 (5 min)[c]	1250		1 (5 min)[c]	1660
1302520	1	1020	1302519	1	1030
1302522	1	1540	1302521	1	945
	1 (5 min)[c]	643		1 (5 min)[c]	1210
1302524	1	1340	1302523	1	2440
	1 (5 min)[c]	738		1 (5 min)[c]	2060
1302526	1	879	1302525	1	1190
1302528	1	565	1302527	1	1150
	1 (5 min)[c]	506		1 (5 min)[c]	576
1302530	1	980	1302529	1	1510
	1 (5 min)[c]	705		1 (5 min)[c]	1020
1302532	1	2440	1302531	1	1080
	1 (5 min)[c]	1160		1 (5 min)[c]	720
1302534	1	1640	1302533	1	1580
	1 (5 min)[c]	1930		1 (5 min)[c]	1040

Mean: 1492; SD: 724; Cut-point (99%): 3176

[a] Control group. [b]Recovery period following the last dose administered on week 6. [c]Week1, 5-min post dose TK samples

Table 3. Immunocapture–LC/MS detection of anti-Protein XYZ ADA (IgG1-4) in monkey plasma from drug-treated groups

Dose mg/kg/wk	Males					Females				
	Sample		LC/MS		ECL	Sample		LC/MS		ECL
	Subject	Time (week)	Peak Area	Result	Result	Subject	Time (week)	Peak Area	Result	Result
5	1302514	1[a]	1140	-	-	1302513	1[a]	1530	-	-
		3	1820	-	+		3	2940	-	+
		6	3750	+	+		6	2400	-	-
	1302516	1[a]	1220	-	-	1302515	1[a]	1400	-	-
		3	4660	+	+		3	3430	+	+
		6	4650	+	+		6	5320	+	+
	1302518	1[a]	745	-	+	1302517	1[a]	1720	-	-
		3	6900	+	+		3	6580	+	-
		6	11600	+	+		6	18800	+	+
50	1302520	1[a]	1020	-	-	1302519	1[a]	1030	-	-
		3	4860	+	+		3	5850	+	+
		6	7020	+	+		6	4720	+	+
	1302522	1[a]	1540	-	-	1302521	1[a]	945	-	-
		3	1800	-	-		3	1090	-	-
		6	3020	-	-		6	1840	-	-
	1302524	1[a]	1340	-	-	1302523	1[a]	2440	-	+
		3	1840	-	-		3	4690	+	+
		6	2190	-	-		6	5540	+	+
100	1302526	1[a]	879	-	+	1302525	1[a]	1190	-	-
		3	10300	+	+		3	1990	-	-
		6	23100	+	+		6	7670	+	+
	1302528	1[a]	565	-	-	1302527	1[a]	1150	-	-
		3	3980	+	-		3	2170	-	-
		6	7210	+	+		6	1380	-	-
	1302530	1[a]	980	-	-	1302529	1[a]	1510	-	-
		3	3020	-	-		3	3520	+	-
		6	7560	+	+		6	3620	+	-
	1302532	1[a]	2440	-	-	1302531	1[a]	1080	-	-
		3	1640	-	-		3	1490	-	-
		6	1700	-	-		6	1580	-	-
		9[b]	2520	-	-		9[b]	1250	-	-
		12[b]	2020	-	-		12[b]	1190	-	-
		15[b]	2900	-	-		15[b]	917	-	-
		18[b]	1830	-	-		18[b]	1080	-	-
	1302534	1[a]	1640	-	-	1302533	1[a]	1580	-	-
		3	8350	+	-		3	20800	+	-
		6	8040	+	+		6	24300	+	+
		9[b]	5180	+	+		9[b]	8930	+	+
		12[b]	5460	+	+		12[b]	6070	+	+
		15[b]	5090	+	+		15[b]	4460	+	+
		18[b]	5490	+	+		18[b]	2240	-	+

[a]Week-1 pre-dose sample also used for cut-point calculation. [b]Recovery period following the last dose administered on week 6, -: ADA negative with LC/MS response < 3176. +: ADA positive with LC/MS response ≥3176

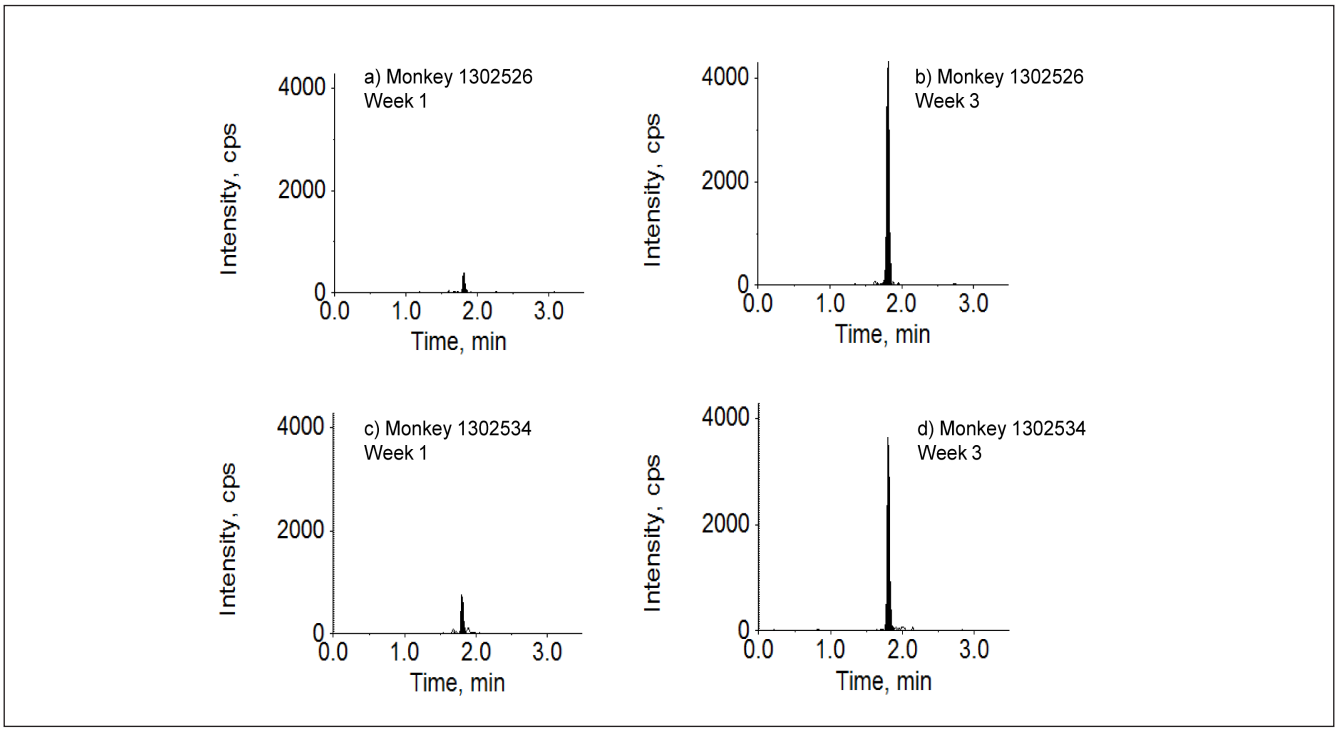

Figure 3. Representative LC/MS chromatograms of surrogate peptide MRM channel m/z 599.33 → 1098.53 from pre-dose (ADA-) and week 3 (ADA+) samples from monkeys 1302526 (a, b) and 1302534 (c, d).

offers an advantage when drug levels in the study samples are high enough such that drug tolerance becomes a concern in other types of assays. It is known that ADA specific to the antigen-binding region of protein drug can interfere with the ability of the drug to bind to the target antigen used in the ELISA method [29]. Thus, the most important element of this approach is that the capture mAb should not compete with ADA for the drug, i.e., the two should not share the same binding domain on the drug. This was assessed for the mouse mAb used for immunocapture during method validation of an immuno-LC/MS human PK assay for Protein XYZ. In the PK assay, Protein XYZ was captured using the same mouse mAb used in the immuno-LC/MS monkey ADA assay, and the resulting drug-mAb complex was then immobilized on magnetic beads, separated from plasma, eluted out from beads, digested, and analyzed by LC/MS. A unique peptide from Protein XYZ was monitored by LC/MS and used to quantitate Protein XYZ. Immunocapture recovery was determined by comparing Protein XYZ concentrations spiked into samples at 10 ng and 100 ng/mL with and without the presence of pre-existing human anti-Protein XYZ antibody. No difference (± 25%) in Protein XYZ concentration was observed (data not shown). In addition, the same mouse mAb was used as immunocapture reagent in an immune-LC/MS human PK assay for a biotherapeutic with a similar construct to Protein XYZ. A monkey ADA positive control was spiked at 4 µg/mL into human plasma containing the biotherapeutic at two lowest calibration levels of 25 and 50 nM to assess potential impact of monkey ADA on the mAb immunocapture recovery. It was found that immunocapture recovery for both standards did not change (± 25%) compared to plasma standards without spiking monkey ADA. It was thus evident that the mouse mAb was indeed able to capture Protein XYZ regardless of whether Protein XYZ was in ADA-Protein XYZ complexes or free form.

The primary goal of this study was to explore the use of LC/MS for detecting monkey ADA, an effort continued from our previous work on LC/MS for detection of pre-existing ADA in human plasma [22]. Assay validation such as accuracy and precision assessment was not part of this preliminary assay development. However, assay validation must be considered, especially in a regulated bioanalytical arena in support of immunogenicity assessments. ADA positive controls and stable isotope labeled peptide internal standard should be used for the assay validation.

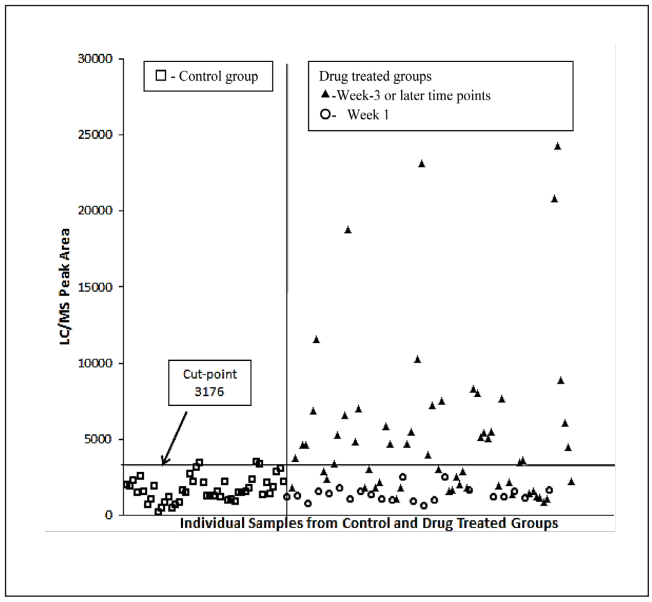

Figure 4. Comparison of LC-MS/MS ADA assay response between the control and drug treated groups

Conclusions

We demonstrated for the first time that immunocapture-LC/MS could be used for detecting cynomolgus monkey anti-Protein XYZ antibody in the presence of high drug levels. In this approach, ADA was converted to drug-ADA complex and then captured using a biotinylated anti-drug mAb on magnetic beads. After separation from plasma using a magnet, the beads were washed and ADA was eluted followed by trypsin digestion and LC/MS analysis of a surrogate peptide unique to monkey IgG1-4. The assay results were in close agreement with those from an ECL immunoassay and were successfully applied in support of a toxicology study. Potential quantitative correlation between these two different approaches may be explored provided that adequate validation is conducted for the LC/MS assay in the future work.

In this study, the immunocapture-LC/MS assay overcame drug interference and thus presents the potential as an alternative technology platform for ADA detection in the presence of high drug levels. The monkey assay was similar to the human ADA assay we have reported [22] but using a monkey-specific peptide for the detection of monkey ADA. Therefore, we demonstrated that LC/MS approach can be used for evaluation of immunogenicity in different species. It should be noted that in order to perform complete assay validation, monkey specific ADA positive control, which was not available for this study, will be needed.

Owing to LC/MS's advantages such as high drug tolerance, high specificity, selectivity and reproducibility, wide dynamic range and multiplexing capability, it is expected that immunocapture-LC/MS will become an additional tool in immunogenicity assessment. Further improvements should focus on reducing endogenous interference and increasing assay sensitivity and specificity. LC/MS assay validation must be considered, especially in the regulated bioanalytical arena in support of immunogenicity assessments, using positive controls and stable labeled peptide internal standard. Species specific ADA positive controls will be needed to perform complete assay validation.

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