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A Fast and Simple Immuno-mass Spectrometry Based Method Enables Universal Preclinical Bioanalysis for IgG-1 Type mAb

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ABSTRACT

Purpose: Establish a generic method for bioanalysis of mAb at preclinical phase with high sensitivity and simple workflow.

Methods: Thermo Scientific™ CaptureSelect™ human IgG-Fc PK biotin conjugate immobilized on Thermo Scientific™ SMART Digest™ IA streptavidin magnetic beads were used for target mAb drug capture and digestion. The resulting surrogate peptides were analyzed by high resolution mass spectrometry with PRM acquisition mode.

Results: We finally established a generic method for bioanalysis of IgG-1 type mAb at preclinical phase using immuno-mass spectrometry, which enables quantification of mAb with high sensitivity in blood of any animal mode in pharmacokinetics studies without any modification. This method is simple and fast to operate, it also enables analyzing the mAb structure integrity *in vivo* when quantification.

INTRODUCTION

Bioanalysis relies on accurate, sensitive, selective and robust quantification of target drugs and their metabolites in kinds of biological matrices. Traditional ligand-binding assays (LBAs) have served as the first choice for protein drug bioanalysis historically, but they suffer from high susceptibility to matrix interference and anti-drug antibody (ADA). Also, the development of LBA is time-consuming.

LC/MS-based methods have emerged as a promising alternative due to its specificity. The vast majority of LC/MS method adopted the bottom-up strategy. However direct bottom-up analysis may not offer enough sensitivity in several cases. Immuno-mass spectrometry method combined the sensitivity offered by immuno-capture and selectivity offered by mass spectrometry. Capture method and signature peptides should be optimized according to different study phase if immuno-mass spectrometry method was employed, and traditional immuno-mass spectrometry method was complicated and time-consuming in daily operation.

Here we report a generic immuno-mass spectrometry method for bioanalysis of IgG-1 type mAb at preclinical phase. We made the workflow fast and simple by using SMART Digest™ IA streptavidin magnetic beads, the total workflow could be finished in 4-5 hours and could be fully automated. We made the method as a universal one by using CaptureSelect™ human IgG-Fc PK biotin conjugate as the capture reagent, this method could be applied to all IgG-1 type mAb in serum of any animal mode in pharmacokinetics studies without any modification. We could also monitor the structure integrity of mAb drug in vivo by analyzing signature peptides in different domains in the constant region. Finally, the LLOQ of this method was 20ng/mL mAb in serum, and this method fulfilled all the criterions of quantification method validation.

MATERIALS AND METHODS

Sample Preparation

SMART Digest IA streptavidin magnetic beads (SDIA beads for short) were bulk prepared with CaptureSelect human IgG-Fc PK Biotin Conjugate (anti-Fc ligand for short) at room temperature for 40 minutes. After one wash with 0.1%BSA/PBS, this anti-Fc beads was resuspended to its original volume with 0.1%BSA/PBS. Each aliquot of 30ul immobilized anti-Fc beads were then added to 50ul of animal serum spiked with various concentrations of IgG-1 type mAb and constant level of internal standard (SILu™Mab K1 from SigmaAldrich), after diluting to the final volume of 200uL with PBS, the sample slurry was incubated at room temperature with gentle mixing for 90 minutes. Non-specific binding proteins were washed away with 0.1%BSA/TBS, 200uL of digestion buffer was then added to the magnetic beads and captured proteins were digested on beads at 70℃ for 60 minutes by gentle shaking. Finally, the supernatant was transferred to a new tube and acidified with 10%FA/50%ACN to the final concentration of 1%FA/5%ACN to fully quenching the reaction.40-80uL of final sample was injected for LC/MS analysis.

LC/MS Method

Peptides were separated through a 10 minutes total gradient using Thermo Scientific™ Accucore™ 150 C18 column (2.6μm pore size, 2.1mm * 50mm) at the flow rate of 0.5mL/min. Mobile phase buf A is 0.1%FA/H_2O, buf B is 0.1%FA/ACN, the instrument used for liquid chromatography is Thermo Scientific™ Vanquish™ Flex UHPLC System.

Data was acquired using PRM mode on Thermo Scientific™ Orbitrap IDX™ mass spectrometry. Slens-RF was set to 50, isolation width for precursor ion was set to 1.4Th, HCD was used for precursor fragmentation and the resolution of MS/MS was set to 30,000. The information of signature peptides and the transitions were listed below.

Table 1. Information of signature peptides and transitions

	Sequence	Signature peptide (light)				Internal standard		NCE	RT (min)
		Precursor (m/z)	Quantitative ion (m/z)	Qualitative ion (m/z)	Ion Ratio	Precursor (m/z)	Fragmental ion (m/z)		
Peptide 2	ALPAPIEK	419.756	486.292 ($y4^+$)	327.695 ($y6^{2+}$)	0.499	423.762	494.305 ($y4^+$)	29	4.04
Peptide 3	GPSVFPLAPSSK	593.828	699.405 ($y7^+$)	846.473 ($y8^+$)	0.710	597.834	707.417 ($y7^+$)	25	5.13
Peptide 5	DSTYSLSSTLTLSK	751.887	1036.589 ($y10^+$)	836.474 ($y8^+$)	0.841	755.891	1044.602 ($y10^+$)	25	5.23
Peptide 7	TTPPVLDSDGSFFLYSK	937.967	836.918 ($y15^{2+}$)	397.208 ($y3^+$)	0.144	941.973	840.924 ($y15^{2+}$)	31	6.05

Data Analysis

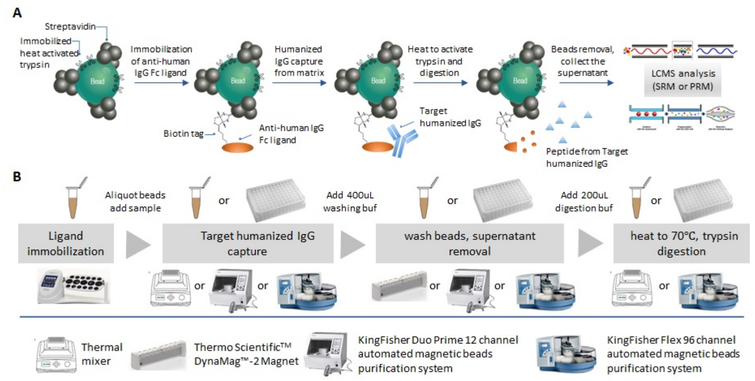
Mass spectrometry data was processed using Thermo Scientific™ TraceFinder™ 4.1 general quan version.

RESULTS

The immuno-mass spectrometry workflow based on SMART Digest IA kit

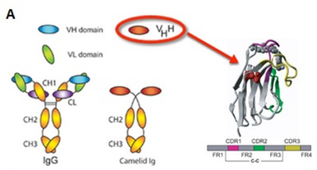
SMART Digest IA streptavidin magnetic beads are the kernel of this immuno-mass spectrometry method. Streptavidin and heat activated trypsin were co-immobilized on the same bead. The SMART Digest trypsin was modified through genetic engineering, it obtained the protein digestion activity only when the temperature raised up to 70°C, and then digested the target proteins captured on the beads. Only four steps were needed for sample preparation as listed in Fig 1. No detergent or denaturant was needed as we used heat to denature the target protein. The total workflow could be finished in 4 hours, as frequent sample transferring, protein denaturing, reduction/alkylation and subsequent SPE step was avoided. This SMART Digest IA workflow could be fully automated via using Thermo Scientific™ KingFisher™ magnetic beads purification system, further increasing the throughput and robustness of sample preparation.

Figure 1. The diagram showing the principle and operation step of immuno-mass spectrometry method using SMART Digest IA streptavidin magnetic beads



For immuno-mass spectrometry method in preclinical bioanalysis of mAb drug, we aimed to capture humanized IgG from the animal serum matrix. CaptureSelect human IgG-Fc PK biotin conjugate (anti-Fc ligand for short) was chosen as the capture reagent. This anti-Fc ligand was composed of the fragment belonging to the variable region of camelid IgG, with the size of 13KDa. It recognized the Fc domain of all human IgG subtypes, but did not react with IgG of animal models used in preclinical studies. 30uL of SMART Digest IA beads offered the capacity of up to 4ug of this anti-Fc ligand.

Figure 2. Information of CaptureSelect human IgG-Fc PK biotin conjugate. A. Structure of human IgG and camelid IgG; B. Binding selectivity of human IgG-Fc biotin conjugate; C. Species reactivity of human IgG-Fc biotin conjugate; D. Anti human IgG-Fc ligand capacity of SDIA beads calculated by measuring the free anti-Fc ligand in the supernatant using PRM. method.



B

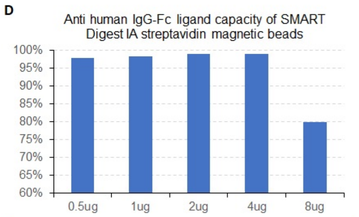
Binding selectivity Human IgG-Fc PK Biotin

Antibody target	Isotype/subclass	Binding selectivity
Ab fragments	Human IgG Fc	✓
	Human IgG Fab	–
IgG subclasses	Human IgG1	✓
	Human IgG2	✓
	Human IgG3	✓
	Human IgG4	✓
Ab isotypes	Human IgGA	–
	Human IgGM	–

C

Antibody target	Isotype/subclass	Binding selectivity
Species	Chimpanzee	✓
	Cynomologus macaque and Rhesus macaque	–
	Rat, Mouse	–
	Sheep, Goat	–

D

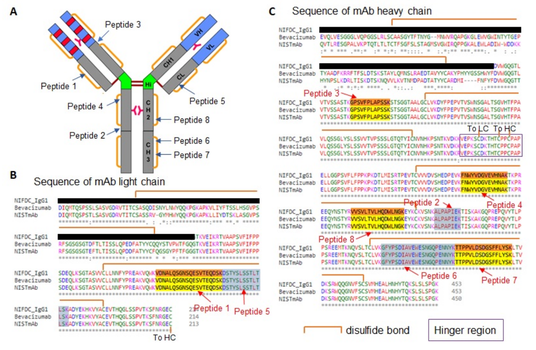


	Quantitative ion	Confirming ion
Peptide 1 of anti-Fc ligand	442.707 -> 696.328	442.707 -> 565.288
Peptide 2 of anti-Fc ligand	628.699 -> 688.373	628.699 -> 560.315

On-bead digestion and signature peptides determination

Digestion efficiency was different if on-bead digestion was compared to in-solution digestion, so we should determinate the appropriate signature peptides based on the on-bead digestion after immuno-capture. The signature peptides should fulfil the criterions below: (1) located in the constant region, and completely conserved among all IgG-1 type mAb drug; (2) strong and stable PRM signal upon on-bead digestion after immuno-capture; (3) recovery of on-bead digestion should be similar compared to in-solution digestion; (4) no matrix interference towards the chosen transition in matrix blank sample; (5) cover more domains in the constant region.

Figure 3. Multiple sequence alignment of humanized IgG-1 type mAb and candidates of signature peptides.



8 candidates of signature peptides were chosen for evaluation, they were completely conserved among humanized IgG-1 type mAbs commercially available (Fig. 3). Finally, 4 signature peptides were selected, 1 in CL domain, 1 in CH1 domain, 1 in CH3 domain and 1 between CH2 and CH3 domain to quantify and also monitor the structure integrity of the target mAb protein (Table 1).

Quantification performance

4 signature peptides showed good linearity in all animal serum matrices. The linearity range of peptide 2,3 and 7 is 39.063 – 10,000 ng/mL, and for peptide 5 is 78.125 – 10,000 ng/mL. The LLOQ of peptide 2 and 3 is 20ng/mL, for peptide 5 and 7 is 50 and 30 ng/mL respectively (Table 2).

Quantification precision and accuracy fulfilled the criterion of methodology validation (Table 3). Finally, peptide 3 was chosen as the quantitative peptide to report the final concentration of target mAb in matrix, while peptide 2, 5, 7 were used as monitoring peptide to investigate the structure integrity of target mAb *in vivo*.

Table 2. Standard curve range, r^2 and LLOQ

Sequence		Linearity range (ng/mL)	r^2, linear fit, $1/x^2$ weight	LLOQ (ng/mL)	Internal standard CV%
Peptide2	ALPAPIEK	39.063 - 10,000	0.996	20	4.50
Peptide3	GPSVFPLAPSSK	39.063 - 10,000	0.993	20	3.34
Peptide5	DSTYSLSSTLTLSK	78.125 - 10,000	0.994	50	4.51
Peptide7	TTPPVLDSDGSFFLYSK	39.063 - 10,000	0.998	30	4.54

Table 3. Quantification precision and accuracy of QC samples

	QCL (100 ng/mL)			QCM (800 ng/mL)			QCH (6,000 ng/mL)		
	Intra CV% (n = 6)	Inter CV% (n = 4)	Diff %	Intra CV% (n = 6)	Inter CV% (n = 4)	Diff %	Intra CV% (n = 6)	Inter CV% (n = 4)	Diff%
Pep2	2.26	2.42	2.11	0.87	1.91	3.44	2.08	2.09	0.52
Pep3	2.87	3.53	2.83	0.98	1.89	2.22	0.87	1.64	-0.08
Pep5	6.54	10.40	-1.51	6.47	7.06	9.54	4.96	8.66	9.30
Pep7	4.28	6.08	7.63	1.50	3.03	9.45	3.70	5.38	1.47

CONCLUSIONS

Benefits of our immuno-mass spectrometry workflow for universal preclinical bioanalysis of IgG-1 type mAb

- Fast and simple, 4-5 hours workflow for total sample preparation, can be fully automated
- Universal method, can be applied to all IgG-1 type mAb in all animal serum matrix at preclinical phase without any modification
- Monitoring mAb structure integrity *in vivo* when quantification
- High sensitivity and selectivity, LLOQ as low as 20ng/mL using only 50uL of serum sample

TRADEMARKS/LICENSING

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