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Pre-clinical estimation of cetuximab using nano-surface and molecular orientation limited (nSMOL) proteolysis and LC-MS/MS

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1. Overview

Monoclonal antibodies (mAbs) have become a major therapeutic strategy in cancer care. As various market reports and sales data suggest, biologics is a way forward for every pharmaceutical organization, inclined towards high profitability. Monoclonal antibody based drug forms the biggest pipeline for both innovator and biosimilar drugs.

For quantification of therapeutic mAbs in pharmacokinetics study, ligand binding assays such as Enzyme-Linked Immuno-Sorbent Assay (ELISA) are the most widely used techniques. However, the major drawbacks of these are influence of cross-reaction and inhibitory materials. In contrast, with LC-MS/MS, analysis is performed based on the structural information; thus, the aforementioned issues can potentially be resolved.

The LC-MS/MS analysis of high-molecular-weight proteins, such as monoclonal antibodies, is normally performed after digesting the protein into smaller peptides using a protease, such as trypsin. However, this process also generates a large number of peptides including the signature peptides. These peptides increase the background noise and ionization suppression, thereby becoming a major cause of data instability. nSMOL, on the other hand ,can decrease these issues by selective proteolysis of the monoclonal antibodies. Therefore, the use of this approach can improve the reproducibility and robustness of the method while maintaining specificity of antibodies.

2. Introduction

nSMOL works on selective proteolysis of Fab region by making use of difference in size of the protease nanoparticle diameter (200 nm) and the antibody resin pore size (100 nm) as shown in Figure 1. To achieve limited proteolysis of the antibody Fab region, the antibody is immobilized in such a way that only Fab region of antibody is spatially available for selective cleavage with protease (trypsin) immobilized on beads. Moreover, effective Fab proteolysis is possible under non-denaturing physiological condition[1],[2],[3]. Thus, there is a considerable reduction of the digested peptides that are formed which enhances selectivity and drastically reduces time required for method setup and optimization. In other words, using nSMOL, one can maintain the specificity of the antibody sequences while minimizing the sample complexity as well as the elimination of extra protease.

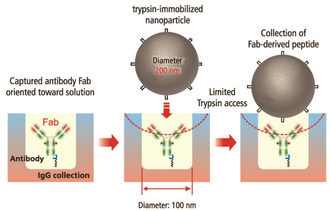

Figure 1. Concept of nSMOL

Cetuximab is a recombinant, human/mouse chimeric monoclonal antibody that binds specifically to the extracellular domain of the human Epidermal Growth Factor Receptor (EGFR). Cetuximab is composed of the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions and has an approximate molecular weight of 152 kDa. It is used in the treatment of metastatic colorectal carcinoma, head and neck cancer. After sample preparation using nSMOL protocol, MRM based quantitation of cetuximab from Wistar rat plasma was carried out using LCMS-8060, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan. nSMOL along with ultra high sensitivity of LCMS-8060 with heated ESI source enabled development of quantitation method for this drug molecule with good accuracy and precision even in presence of complex matrix like rat plasma.

3. Methods

3-1. Sample preparation

Cetuximab was spiked in Wistar rat plasma over a concentration range of 0.29 to 150 µg/mL for linearity study[4]. Two sets of LLQC (0.29 µg/mL) , MQC (4.69 µg/mL) and HQC (75 µg/mL) samples were also prepared in Wistar rat plasma. All the calibration standards and QC samples were processed using nSMOL protocol described in Table 1.

Table 1. nSMOL sample preparation protocol

Add immunoglobulin collection resin, wash solution 1 and plasma to sample tube and shake this tube for 15 minutes at room temperature
↓
Transfer the solution to the filter cup and centrifuge
↓
Add wash solution 1 to the filter cup and centrifuge
↓
Add wash solution 2 to the filter cup and centrifuge
↓
Add enhanced reaction solution and FG beads trypsin DART to filter cup and perform proteolysis
↓
Collect the peptides by centrifugation and transfer to the vials for LC-MS/MS analysis

3-2. LC-MS/MS analysis

Cetuximab was analyzed using Ultra High Performance Liquid Chromatography (UHPLC) Nexera X2 coupled with LCMS-8060 (shown in Figure 2).

LCMS-8060, sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

In order to improve ionization efficiency, the newly developed heated ESI probe (shown in Figure 3) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background.

The details of analytical conditions are given in Table 2.

Figure 2. Nexera X2 with LCMS-8060 triple quadrupole mass spectrometer

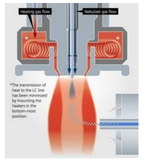

Figure 3. Heated ESI probe

Table 2. Analytical conditions for cetuximab

UHPLC condition (Nexera X2)	
Column	Shim-pack GISS C18
Mobile phase	A: 0.1 % formic acid in water
	B: 0.1 % formic acid acetonitrile
Elution mode	Gradient mode
Column temperature	50 ^{0}C
MS parameters (LCMS-8060)	
MS interface	Electro Spray Ionization (ESI)
Nitrogen gas flow	Nebulizing gas- 3 L/min; Drying gas- 10 L/min
Zero air flow	Heating gas- 18 L/min
MS temperatures	Desolvation line- 200 ^{0}C; Heating block- 400 ^{0}C; Interface- 380 ^{0}C

4. Results

Unique peptide for cetuximab was selected using Skyline (MacCoss Lab, University of Washington). 'GPSVFPLAPSSK' peptide selectively represents cetuximab from rat plasma. However, this peptide is generic signature peptide. Therefore, this method can be selectively used for only assessment of a singular chimeric mAb in rat plasma. MRM transition of 594 > 699 for unique peptide 'GPSVFPLAPSSK' was selected for cetuximab quantitation. Linearity study was carried out using internal standard calibration method. $P_{14}R$ was used as internal standard and MRM transition of 512 > 292 was selected for internal standard peptide. MRM transitions were optimized using automatic MRM optimization feature of LabSolutions.

MRM chromatogram of blank rat plasma is shown in Figure 4. MRM chromatograms of 0.29 µg/mL and 150 µg/mL of cetuximab in rat plasma is shown in Figures 5 and 6 respectively. Calibration graph obtained for cetuximab analysis from 0.29 to 150 µg/mL in rat plasma is shown in Figure 7. Results of calibration standards and quality control samples are tabulated in Table 3.

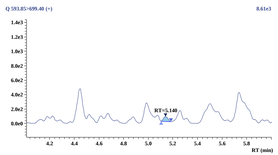

Figure 4. Representative MRM chromatogram of blank Wistar rat plasma

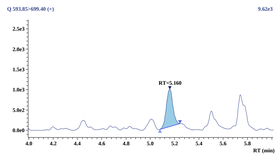

Figure 5. Representative MRM chromatogram of 0.29 µg/mL of cetuximab in Wistar rat plasma

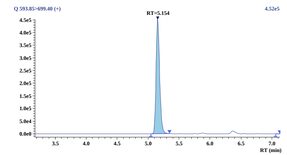

Figure 6. Representative MRM chromatogram of 150 µg/mL of cetuximab in Wistar rat plasma

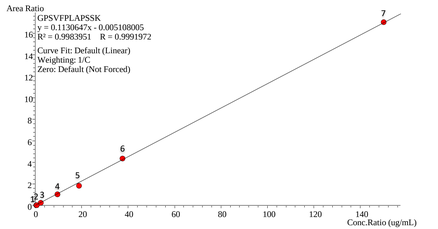

Figure 7. Calibration graph for cetuximab analysis in rat plasma

Table 3. Results of CC and QC analysis of cetuximab

Sample name	Standard concentration (µg/mL)	Calculated concentration from calibration graph (µg/mL)	% Accuracy
Standard 1	0.29	0.35	119.81
Standard 2	0.59	0.50	84.74
Standard 3	2.34	2.43	103.88
Standard 4	9.38	9.35	99.73
Standard 5	18.75	16.51	88.07
Standard 6	37.50	38.65	103.06
Standard 7	150.00	151.06	100.70
LLQC_01	0.29	0.30	104.60
MQC_01	4.69	4.42	94.29
HQC_01	75.00	74.34	99.12
LLQC_02	0.29	0.24	84.35
MQC_02	4.69	4.75	101.25
HQC_02	75.00	77.91	103.89

5. Conclusion

- Cetuximab was analyzed from Wistar rat plasma over a concentration range of 0.29 to 150 µg/mL by using nSMOL protocol and LCMS-8060. Good linearity was observed for this concentration range with R^2 value of 0.9983 and % accuracy for standards as well as for control samples were within a range of 80 to 120 % .
- nSMOL protocol with minimal sample preparation steps helped in quick method development for quantitation of cetuximab from rat plasma.

6. References

[1] Iwamoto N, et al., Analytical methods, Volume 7, Issue 21 (2015), 9177-9183.

[2] Iwamoto N, et al., Analyst, Volume 139, Issue 3, (2014), 576-580.

[3] Iwamoto N, et al., Drug Metabolism and Pharmacokinetics, Volume 31, Issue 1, (2016), 46-50.

[4] Iwamoto N, et al., Bioanalysis, Volume 8, Issue 10, (2016), 1009-1020.