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An Immunoaffinity-High Resolution Accurate Mass Assay for the Pre-Clinical Quantification of Trastuzumab in Rat Plasma

MRMHR using the TripleTOF® 6600 LC-MS/MS system and the IonDrive™ Turbo V source

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Key Challenges in the Quantification of Trastuzumab

- Traditional ELISA-only based techniques can suffer from high variability, narrow dynamic range, and problems with selectivity.

- ELISA-only based assays typically cannot use internal standards which can help to correct for anomalies and ensure accurate quantification.

Key Advantages Using Immunoaffinity Enrichment coupled with MRMHR on the TripleTOF® 6600 System

- A significant increase in selectivity and sensitivity

- Excellent robustness, reproducibility, and dynamic range

- The mode of capture using immunoaffinity can be more generic when paired with LC-MS/MS

- HRAMS MS/MS spectra can be used for confirmation of modifications and isomers

Introduction

LC-MS/MS based assays have seen wide adoption for the quantification of therapeutic peptides and proteins in biological matrices. The typical procedure for a protein therapeutic is enzymatic digestion and quantification of either universal or signature peptides depending on the matrix background. In recent years immunoaffinity sample preparation techniques for sample purification and enrichment before digestion have been widely investigated.[1] There are multiple reasons for the implementation of hyphenated immunocapture-LC-MS/MS. One of the key benefits is the significant increase in selectivity and sensitivity that can be achieved by combining the two techniques in addition to wide dynamic range and excellent robustness and reproducibility.

Figure 1. The SCIEX TripleTOF® 6600 System and ExionLCTM AD System.

Immunoaffinity sample prep techniques can be adapted to different assay requirements and different matrices by changing the mode of capture. The mode of capture chosen for the therapeutic protein can be generic when paired with LC-MS/MS, which can be used to get an assay up and running rapidly, or very specific if required. This allows an assay to be adapted through the lifetime of the therapeutic as it transitions from discovery to the clinic.

The generic approach can be utilized in the pre-clinical setting for fast method deployment. For example, using anti-human IgG (Fc specific) capture antibodies allows for a simple and generic methodology for monoclonal antibodies such as Trastuzumab. In addition, multiple candidates can be rapidly screened using the same capture process.

Routinely, immunoaffinity prepared samples are analyzed by signature peptide quantification on triple quadrupoles. In this study, we present an immunoaffinity high resolution accurate mass (HRAMS) assay as a complimentary analytical technique for signature peptide quantification.

Drug Discovery and Development

The assay performance is measured using Trastuzumab in rat plasma as a case study.

Materials and Methods

Sample Preparation: Sprague Dawley K2 EDTA rat plasma (Seralab, UK) was spiked with Trastuzumab to create a range of calibration standards, quality controls, and blanks. The sample workup utilized streptavidin coated magnetic immunoaffinity enrichment beads. The generic workflow is outlined in Figure 2.

The protocol used follows previously published work for Trastuzumab quantification on the SCIEX QTRAP® 6500+ LC-MS/MS system.[2]

The volume of plasma used during preparation for all samples (standards, QCs, blanks) was 50 μL. Following the immunoaffinity preparation and digestion protocols, 50 μL of the final aliquot was diluted 1:1 with water for LC-HRAMS injection. A calibration range from 5 ng/mL to 50,000 ng/mL was analyzed.

UHPLC Conditions: A SCIEX ExionLC™ AD UHPLC system was used. For the signature peptide quantification a Phenomenex Aeris XB-Peptide, 50x2.1mm, 1.7μm column was used at 30°C. A gradient of mobile phase A; 0.1% formic acid in water and mobile phase B; 0.1% formic acid in acetonitrile was used at a flow rate of 600 μL/min. The injection volume was set to 25 μL. A 3.5 minute gradient elution profile was utilized with a total runtime of 7 minutes.

HRAMS Conditions: A SCIEX TripleTOF® 6600 (HRAMS) LC-MS/MS system equipped with the IonDrive™ Turbo V source was used.

The SCIEX TripleTOF 6600 system was setup with MRMHR acquisition for FTISKADTSK the signature peptide and DTLIMSR (heavy chain IgG1, IgG2, IgG3 & IgG4) as the universal peptide. SILu™MAb was added before sample preparation with heavy labelled DTLIMSR* used as the internal standard. Acquisition method settings (declustering potential and collision energies) were transferred from a method developed on the SCIEX QTRAP 6500+ System.[2]

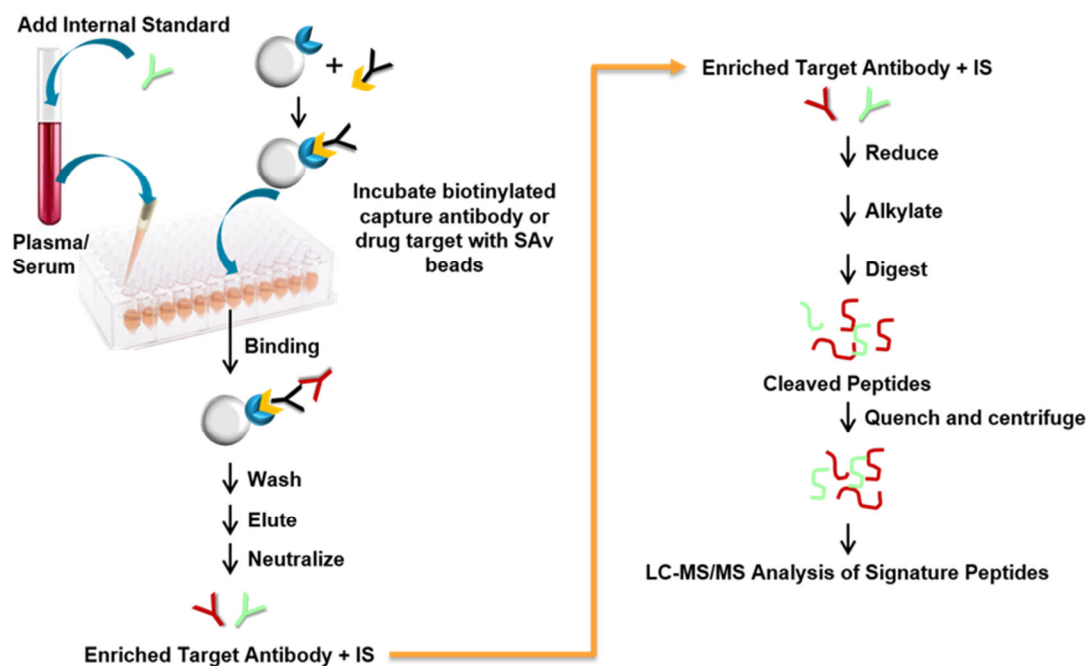**Software:** Analyst® TF software 1.7.1 and MultiQuant™ software 3.0.2 were used.

Figure 2. Immunoaffinity sample preparation workflow.

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Results

The assay selectivity, sensitivity, linear dynamic range, accuracy and precision were all measured for the assay.

The initial performance criteria measured include the number of data points across the LC peak, the quality of the MRMHR spectra and the selectivity of the individual fragment ions. These are shown in Figure 3, in Figure 3a the chromatographic conditions used resulted in

a peak width of approximately 4 seconds with 15 data points across the peak. To achieve this, each experiment (a single TOF MS and three MRMHR experiments) were acquired with a 40 ms accumulation time. In Figure 3b and 3c the TOF MS/MS spectra for FTISADTSK and DTLIMSR are shown, respectively. Here we have high quality MS/MS spectra which have been acquired in MRMHR high sensitivity mode with a mass resolution between 20-23K.
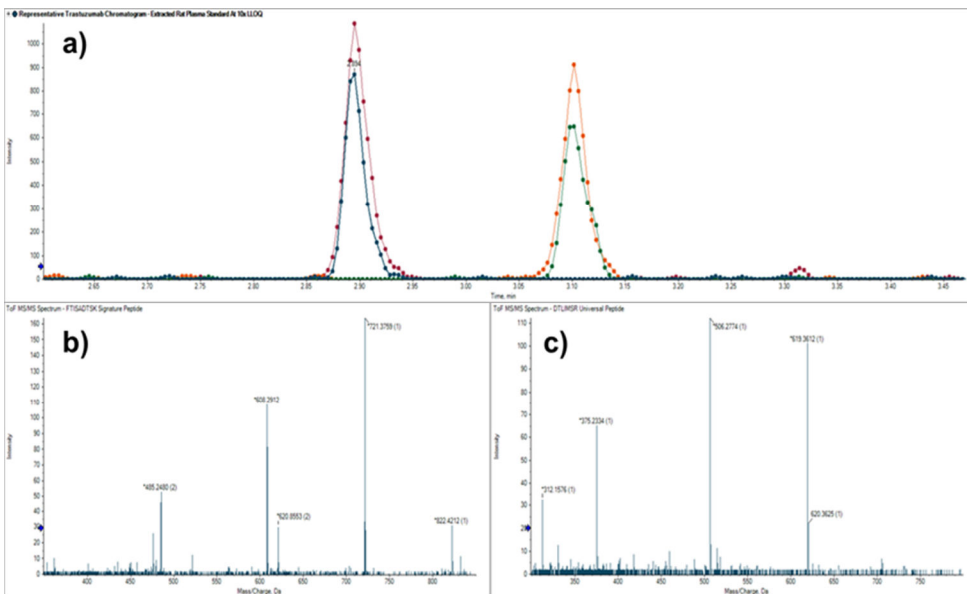

Figure 3. a) Extracted Ion Chromatograms (XIC) for FTISADTSK and DTLIMSR shown with data points, extracted with a 10mDa width. b) TOF MS/MS spectrum for FTISADTSK. c) TOF MS/MS spectrum for DTLIMSR.
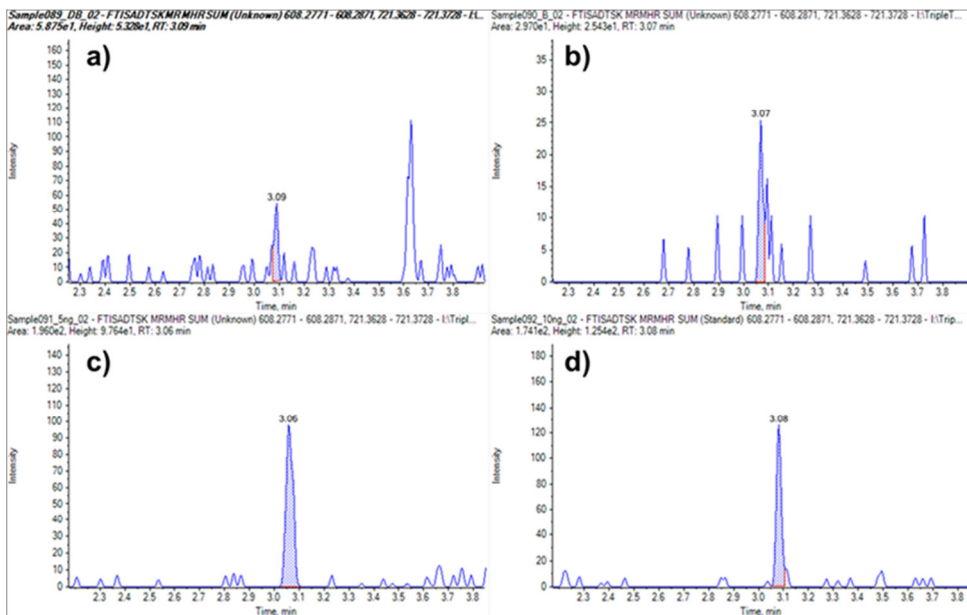

Figure 4. a) Double blank XIC for FTISADTSK. b) Blank for FTISADTSK. c) 5ng/mL Standard for FTISADTSK. d) 10ng/mL Standard for FTISADTSK.
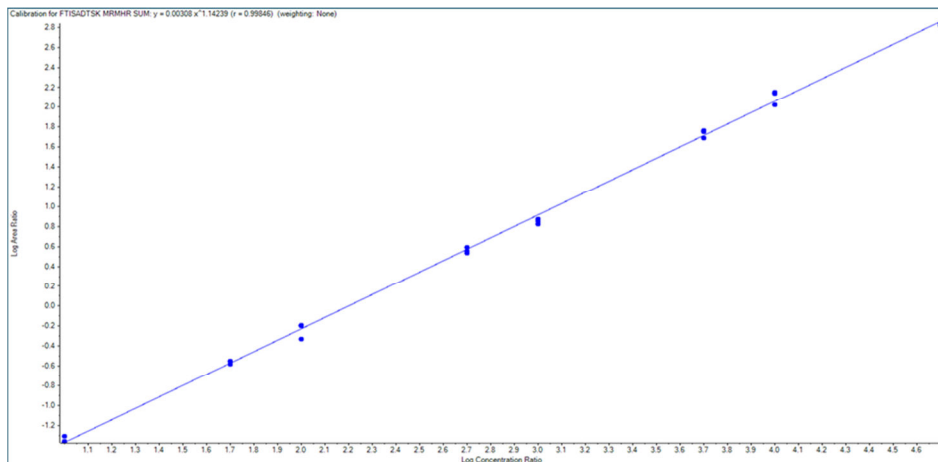
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Figure 5 and Table 1. Calibration curve and associated statistics for accuracy and precision.

	Concentration (ng/mL)								
	5	10	50	100	500	1000	5000	10000	50000
Ave. Measured Concentration (ng/mL)	LOD	10.6	51.0	98.2	491	881	5279	11022	46920
Accuracy (%)	LOD	105%	102%	98%	98%	88%	106%	110%	94%
Precision (%CV)	LOD	6.5%	4.7%	15.1%	5.8%	5.3%	6.7%	13.7%	2.5%

Figure 6 and Table 2. QC sample and associated statistics for accuracy and precision.

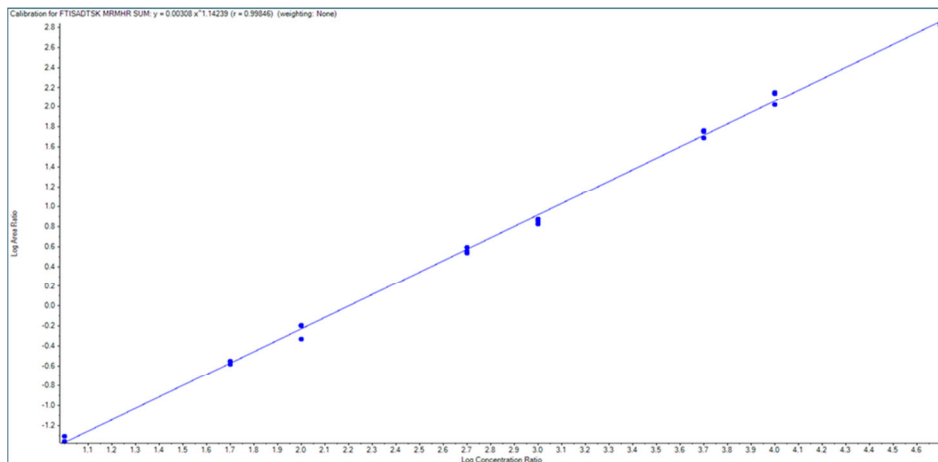|	Concentration (ng/mL)			
	25	62.5	625	6250
Average Measured Concentration (ng/mL) n=8	24.4	58.1	628.5	6040
Accuracy (%)	98%	93%	101%	97%
Precision (%)	11.7%	13.4%	6.7%	6.6%

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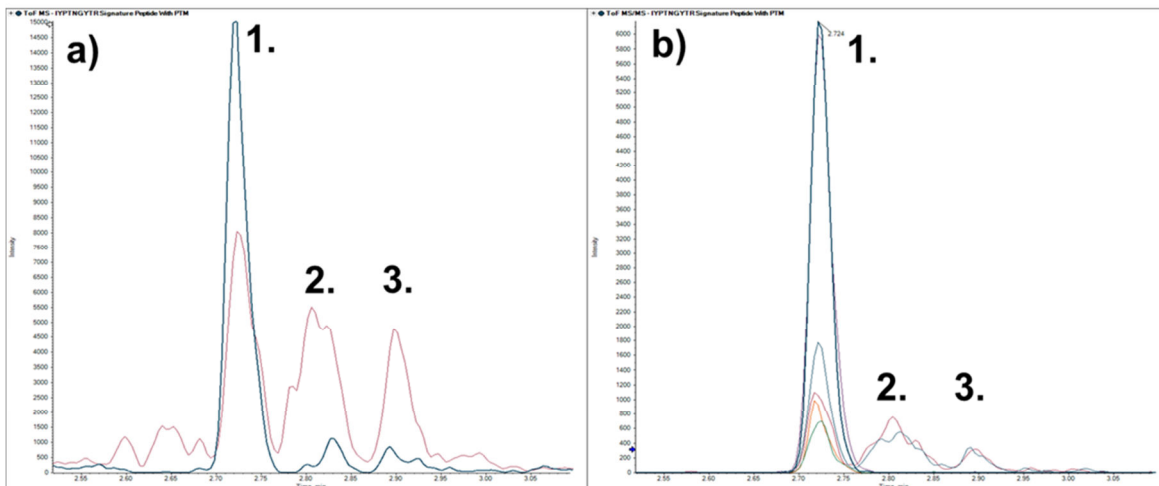
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Figure 7. a) TOF MS XIC for IYPTNGYTR and deamidation products. b) TOF MS/MS XIC for 1. IYPTNGYTR and 2. 3. deamidation products.
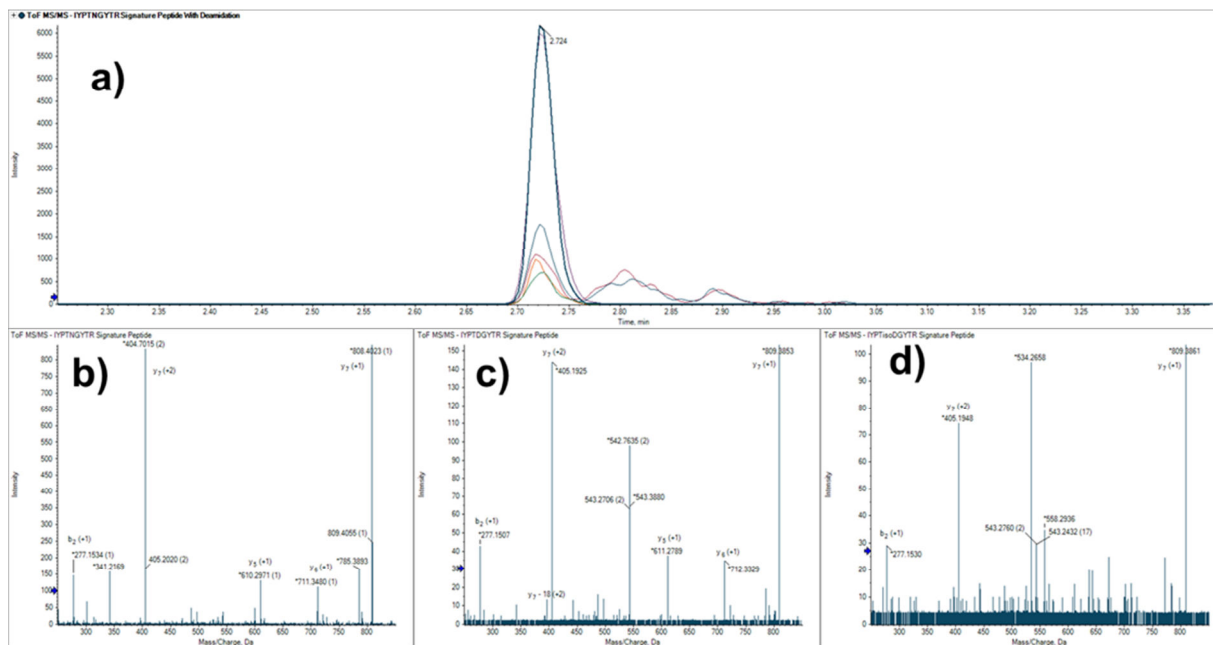

Figure 8. a) TOF MS/MS XICs for IYPTNGYTR and deamidation products. b) MS/MS spectrum IYPTNGYTR. c) MS/MS spectrum for IYPTDGYTR. d) MS/MS spectrum IYPTisoDGYTR.

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The next analytical criteria to be tested included; sensitivity, linear dynamic range, accuracy and precision. This was tested by the acquisition of a small batch of standards and quality controls (QC's) at 2.5xLLOQ, low, medium and high concentration.

Figure 4 shows the chromatograms for the signature peptide (FTISADTSK) for the double blank, blank, 5 ng/mL and 10 ng/mL extracted rat plasma samples.Figure 5 shows the calibration curve achieved across the desired range of 5 ng/mL to 50,000 ng/mL. As can be seen the immunoaffinity LC-HRAM MS/MS assay shows good linear range with an r value of 0.9984. Table 1 shows the accuracy and precision for triplicate injections at each of the standard concentrations. As can be seen from Table 1, the assay performs within recommended acceptance criteria values with accuracy of the calibration standards between 88% and 110% and percentage co-efficient of variation (CV) 15% or less. The assay shows approximately 4 orders of linear dynamic range with linearity still observed at the highest concentration level.

Figure 6 and Table 2 show the bracketed quality controls at 2.5x LLOQ, low, medium and high concentration (25, 62.5, 625, and 6250 ng/mL), a, b, c, and d respectively.

As can be seen good performance is observed in the QC samples with accuracy within 98% and 101% and precision less than 15% CV.

HRAMS Added Value

A key advantage for analyzing quantitative assays with HRAMS is the additional information that can be drawn out of a dataset. The Asn55 containing peptide IYPTNGYTR is an alternative signature peptide that is routinely used for the analysis of Trastuzumab. This peptide can form an in vitro and in vivo deamidation product with additional concomitant isomerization. This forms the aspartic acid peptide (IYPTDGYTR) and the iso-aspartic acid isomer (IYPTisoDGYTR).[3]

Figure 7 (Page 6) shows the TOF MS and TOF MS/MS extracted ion chromatograms for the three peptides. As can be seen the aspartic and iso-aspartic acid peptides can be observed at 2.83 and 2.91 minutes, respectively.

To confirm the identity of each observed peak the MS/MS spectra are compared back to the known sequence.

As shown in Figure 8 (Page 6) the y and b ion series for each of the peptides has been matched to the experimental MS/MS.

Conclusions

In this work we have described how an immunoaffinity-HRAMS assay with the TripleTOF 6600 system can meet the demands of a pre-clinical biotherapeutic assay. The assay shows excellent sensitivity with limits of detection and quantification at 5 ng/mL and 10 ng/mL respectively.

Furthermore the immunoaffinity-HRAMS assay as implemented on the TripleTOF 6600 has the added value of enabling the ability to search and confirm both in vitro and in vivo peptide modifications using the MRMHR MS/MS data.

References

1. E. Fung, P. Bryan & A. Kozhich. Techniques for quantitative LC-MS/MS analysis of protein therapeutics: advances in enzyme digestion and immunocapture. Bioanalysis, Vol. 8, No. 8, Review

2. I. Moore et al. A Hybrid Immunoaffinity-LC-MS/MS Method for Quantifying Trastuzumab in Rat Plasma Using The SCIEX BioBA Solution. RUO-MKT-02-5225-A

3. K. Bronsema, J. Causon et al. HRAMS Monitoring of In-Vivo Protein Biotransformations. ASMS 2017 Poster - RUO-MKT-10-5823-A

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