TWO DIMENSIONAL LC-SRM ASSAY FOR A THERAPEUTIC MONOCLONAL ANTIBODY (TRASTUZUMAB) IN HUMAN SERUM

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OVERVIEW

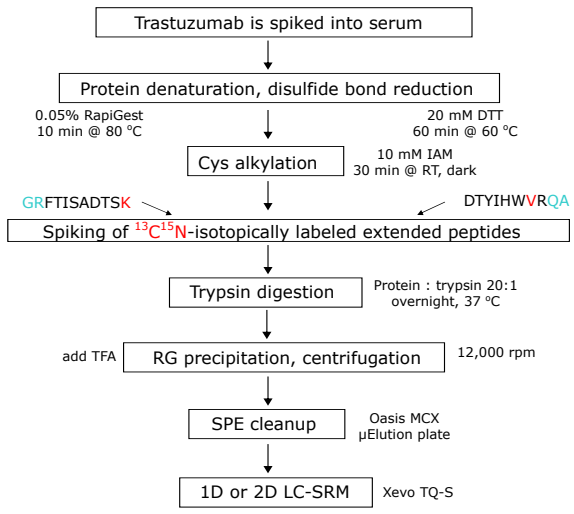
A 2D LC-SRM assay involving no protein prefractionation was developed for quantification of trastuzumab in human serum.

INTRODUCTION

Quantification of therapeutic proteins in serum without analyte prefractionation can offer some advantages in terms of reducing the assay costs and simplifying the sample preparation workflow. Analyte isolation (typically performed by immunoaffinity) has additional purification steps and requires more expensive isotopically labeled protein standards to account for analyte recovery.

Extended $^{13}C^{15}N$-isotopically labeled peptides have been proposed as IS in the case of unfractionated serum samples. One concern in this approach is related to the ability of the digestion enzyme to cleave with the same efficiency the therapeutic protein as well as the isotopically labeled peptide IS in the presence of the serum background. Another concern with the "whole digest" approach is related to analyte/IS suppression in the presence of the serum peptide background. Here we demonstrate that, after a few trypsin digestion optimization steps, extended $^{13}C^{15}N$-peptides can be used successfully for quantification of therapeutic proteins in serum. In addition, we show that two dimensional high pH/low pH RP/RP chromatography is able to reduce significantly ion suppression in protein bioanalysis.

WORKFLOW OVERVIEW



METHODS

Sample Preparation

Trypsin optimization protocol

The analyte (trastuzumab, 150 kDa mAb) was spiked into 40 µL of human serum to reach a final concentration of either 5 nM or 25 nM in the digested sample. The sample was denatured with 0.05% RapiGest at 80 °C for 10 min, reduced with 20 mM dithiothreitol (DTT) for 60 min at 60 °C and alkylated with 10 mM iodoacetamide (IAM) for 30 min at room temperature in the dark. Two extended $^{13}C^{15}N$-isotopically labeled peptides (GRFTISADTSK and DTYIHWVRQA) were spiked in the sample such that the final concentration in the digest was either 5 nM or 25 nM for each peptide. Several protein:enzyme digestion ratios were investigated along with different incubation times using Sigma porcine trypsin (cat no T-6567). Following digestion, 100 µL of sample were diluted 1:1 with a solution containing 4% H_3PO_4 and loaded on an Oasis® MCX mixed-mode µElution plate. Digests were eluted with 2 x 50 µL aliquots of 25% ACN in 2% NH_4OH (pH 10).

Evaluation of signal suppression

Trastuzumab and human serum were digested independently following the protocol described above. The trastuzumab digest was spiked into the SPE cleaned serum digest (1 to 20 dilution) to reach a concentration of 5 nM trastuzumab digest and 10 nM of $^{13}C^{15}N$ peptides.

LC/MS Conditions

1D LC-SRM

An ACQUITY UPLC® I-Class system (Waters) equipped with a BEH300™ C_{18} column (2.1 x 150 mm, 1.7 µm, P/N 180003687) was used. The column temperature was maintained at 35 °C and the flow rate was 0.3 mL/min. Mobile phases contained 0.1 % (v/v) formic acid (FA) in water (A) and 0.1% (v/v) FA in acetonitrile (B). Peptides were eluted with a linear gradient from 0 to 35% B in 10 min.

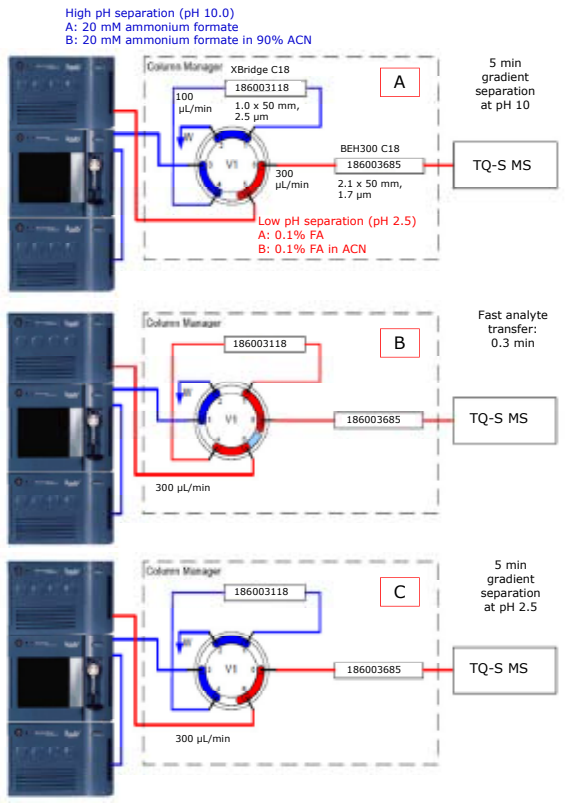
2D LC-SRM

An ACQUITY UPLC® I-Class system with 2D technology (Waters) was used for peptide separations. A reversed-phase/reversed-phase (RP/RP) method was developed that uses the pH of the mobile phases to change the selectivity of a peptide separation in two separate dimensions [1,2]. The diagram and the operating conditions of the 2D-LC system are presented in Figure 1.

SRM Assay

SRM analyses were performed on a Xevo™ TQ-S mass spectrometer. Operating parameters: ESI potential 3.5 kV, CV 30 V, source 120 °C, MS1/MS2 isolation window 0.75 Da (FWHM), adjustable collision energy depending of precursors/fragment (15-30 ev range) and 50 ms dwell time.

Xevo TQ-S tandem quadrupole mass spectrometer.



Time Range	Valve Position	Analyte undergoes
0 - 6.6 min	1	high pH RP separation
6.6 - 6.9 min	2	transfer from 1 D to 2D
6.9 - 14 min	1	low pH RP separation

Figure 1: Heart cut configuration for 2-dimensional chromatography: (A) Sample loading and first dimension separation under basic conditions (pH 10.0); (B) Analyte transfer from the first dimension to the second dimension; (C) Separation in the second dimension under acidic conditions at pH 2.5.

First Dimension separation (pH=10):
Column (P/N 186003118): XBridge C_{18}, 1.0 x 50 mm, 2.5 µm particles, operated at 100 µL/min, kept at 35 °C
Mobile phases: 20 mM ammonium formate in water (Solvent A)
20 mM ammonium formate in 90% ACN (Solvent B)
Gradient profile: from 0 to 40% B over 5 min

Second Dimension separation (pH=2.5):
Column (P/N 186003685): BEH300, 2.1 x 50 mm, 1.7 µm particles,
Mobile phases: 0.1% formic acid in water (Eluent A)
0.1% FA in ACN (Eluent B)
Gradient profile: from 0 to 30% B in 5 min (starts 7 min after inj.).

RESULTS

Trypsin Digestion Optimization

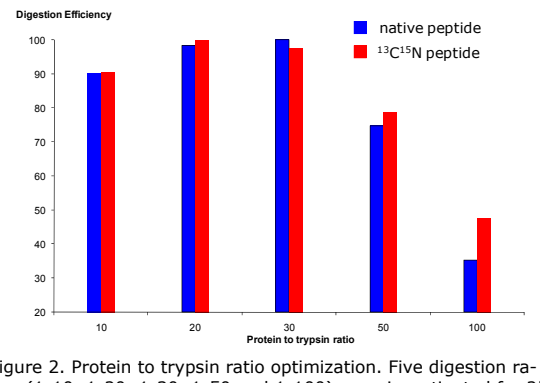


Figure 2. Protein to trypsin ratio optimization. Five digestion ratios (1:10, 1:20, 1:30, 1:50 and 1:100) were investigated for 25 nM trastuzumab and 25 nM $^{13}C^{15}N$-isotopically labeled peptides digested in human serum. The samples were digested overnight (16h) with Sigma trypsin (cat no T-6567) at 37 °C.

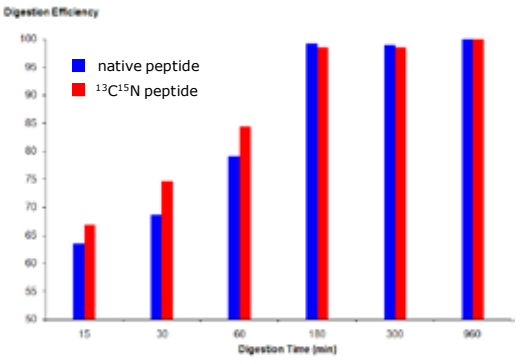


Figure 3. Effect of incubation time on digestion efficiency. Trastuzumab (25 nM) and $^{13}C^{15}N$-isotopically labeled peptides (25 nM) were digested in serum for 15 min, 30 min, 1h, 3h, 6h and 16 h (overnight) with Sigma trypsin (cat no T-6567) at 37 °C.

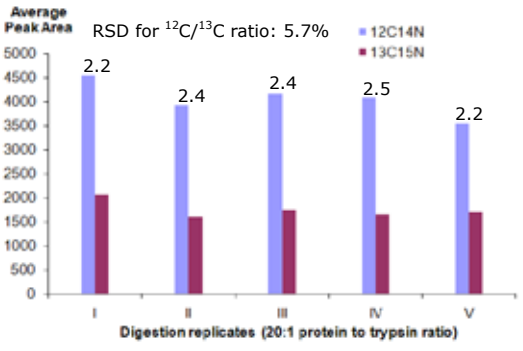


Figure 4. Reproducibility of trypsin digestion. Serum was spiked with 5 nM trastuzumab and 5 nM $^{13}C^{15}N$-isotopically labeled peptides and digested overnight (16 h) with Sigma trypsin (T-6567) at 37 °C. The RSD % for the $^{12}C/^{13}C$ peptide ratio was better than 6%.

2D LC-SRM Assay

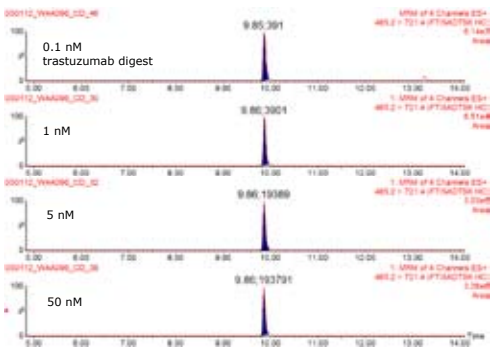


Figure 5. Linearity of the 2D-SRM assay. Four digest concentrations covering a dynamic range of 500 fold (0.1 to 50 nM trastuzumab) were prepared in 20 mM ammonium formate (pH 10) and analyzed in replicate (n=4). The RSD for this assay was better than 2%.

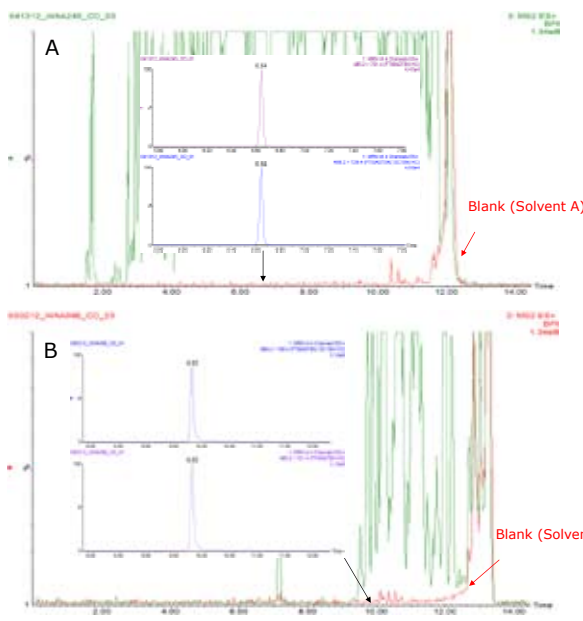


Figure 6. RADAR monitoring of SPE-cleaned samples. (A) 1D LC-SRM separation under acidic conditions (pH 2.5); (B) 2D LC-SRM separation with heart cutting. The sample was prepared by spiking a trastuzumab digest (5 nM trastuzumab and 10 nM $^{13}C^{15}N$ peptides) into SPE-cleaned human serum digest.

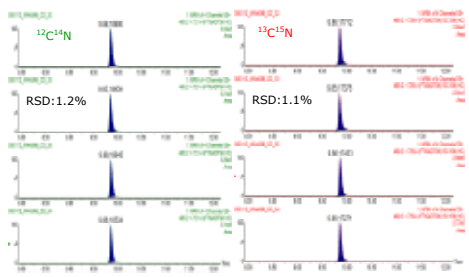


Figure 7. Reproducibility of the 2D LC-SRM assay in the absence of the serum background. A sample containing 5 nM trastuzumab and 10 nM $^{13}C^{15}N$ peptides was prepared in 20 mM ammonium formate (pH 10). Peak area RSD was < 2.0%.

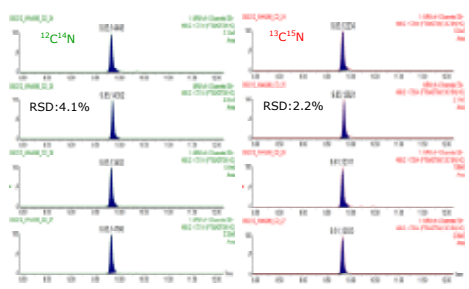


Figure 8. Reproducibility of the 2D LC-SRM assay in the presence of the serum digest. A sample containing 5 nM trastuzumab and 10 nM $^{13}C^{15}N$ peptides was spiked into SPE-cleaned human serum digest. Peak area RSD was better than 5.0%.

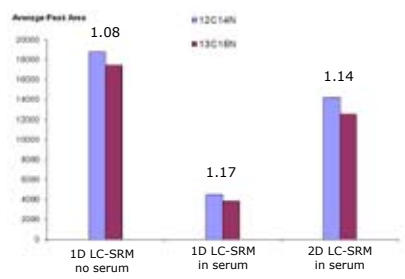


Figure 9. Evaluation of signal suppression. Comparison of average peak areas recorded for the endogenous and isotopically labeled trastuzumab peptide FTISADTSK in the absence/presence of the serum digest matrix. A sample containing 5 nM trastuzumab digest and 10 nM $^{13}C^{15}N$ peptides was spiked into SPE-cleaned human serum digest.

CONCLUSIONS

- A general protein bioanalysis workflow involving no protein fractionation was used successfully for quantification of trastuzumab in human serum.
- Trypsin digestion efficiency for trastuzumab and peptide IS were optimized in respect to protein:enzyme ratio and incubation time in the presence of serum matrix.
- Digestion reproducibility was better than 6% when using an extended $^{13}C^{15}N$-isotopically labeled peptide as an internal standard.

References

1.Gilar M, Olivova P, Daly AE, Gebler JC *J. Sep. Sci*, **2005**, 1694.
2.Doneanu et al *mAbs Journal*, **2012**, 4:1, 24.