A Challenge in the Application of Hybrid Affinity Mass Spectrometry for the Analysis of Peptides

David Woods, Kean Woodmansey and Iain Love
Charles River, Edinburgh, UK

charles river

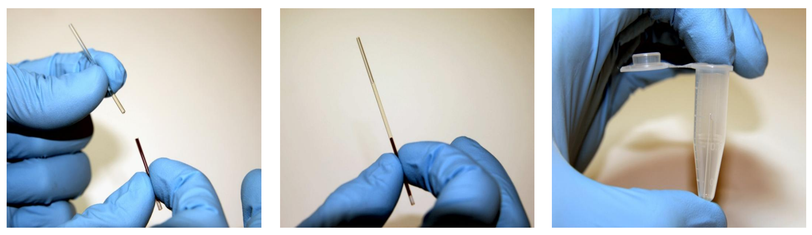
1 INTRODUCTION

Peptides are a class of compounds comprised of short chains of amino acid monomers linked by amide bonds. There is a significant degree of interest in peptide or peptide-like molecules as potential drug candidates owing to their high specificity when compared with small molecules. Additionally, peptides are (generally) non-(immuno)toxic, can be conjugated to deliver toxic payloads and have a higher success rate in Clinical studies than small molecule candidates.

This poster describes a First-in-Man programme for a 39-mer therapeutic peptide candidate. The peptide candidate had two significant Human metabolites relating to a loss of 4 and 5 amino acids respectively from the N-terminal of the amino acid chain.

In the early phases of the programme there was a desire to minimise the number of test subjects used for TK in small preclinical species. A capillary plasma microsampling approach was employed (see Figure 1).

Figure 1: Capillary Plasma Microsampling



2 PRECLINICAL DEVELOPMENT

Peptides present a number of challenges to the Bioanalytical scientist with handling (solubility, adsorption, and stability), chromatography and mass spectrometry often requiring careful and unique optimisation. In this instance, the volume limitation imposed through a microsampling approach exacerbated the sensitivity challenges of a broad peptide charge envelope. Furthermore, non-selective sample preparation is ill-advised where sensitivity is a challenge owing to the potential for matrix effects. To this end a microelution ion-exchange solid phase extraction incorporating a protein binding disruption step was developed (see Table 1).

Table 1: Preclinical Sample Preparation

Step	Reagent
Pre-treatment	4M cold Urea/20mM NH_4OAc
Load onto ionized SPE	4M cold Urea/20mM NH_4OAc
Wash	20 mM NH_4OAc
Elute	MeCN/H_2O/TFA (80/20/0.5)
Dilute	Aqueous

Chromatographic retention and separation was afforded in reverse phase using a charged surface hybrid stationary phase. Adequate sensitivity was achieved using an API6500 mass spectrometer. Each peptide was quantified from an M+4H or M+5H primary ion with secondary ions in lower charge states.

Following development, the assay was successfully validated in 3 preclinical species in support of a full IND enabling toxicology package.

3 CLINICAL ASSAY

Moving into the Clinic a 500-1000x reduction in the LLOQ of the bioanalytical assay was required. A simple means of achieving a lower LLOQ in Clinical assays is to scale up the analytical matrix volume relative to the preclinical assay. In this instance the improvements in LLOQ were outside the scope of matrix volume adjustment. Moreover, this approach also carried a risk of introducing a matrix effect.

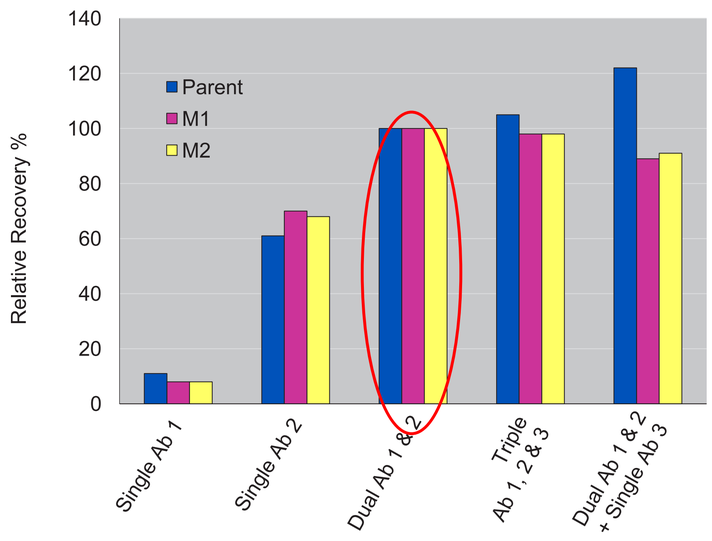
Owing to these limitations a biological affinity based sample preparation was considered as a means to combine selective sample clean-up with selective MS/MS detection.

Biological affinity sample preparation techniques involve the use of analyte directed capture antibodies to selectively bind to a specific epitope on the target analyte. Often involving a solid support, affinity approaches are common place in the preparation of samples for MS/MS analysis of protein drug candidates or biomarkers.

A small library of monoclonal antibodies were raised against the target peptide for a proposed ELISA endpoint but found to be non-specific owing to the presence of the two metabolites. Owing to the mass selectivity afforded by MS/MS it was postulated that the antibodies could be utilised in a selective clean up ahead of analysis by LC-MS/MS. A process was developed to graft three capture antibody candidates to streptavidin coated Dynabeads™.

Three mAbs were identified, each with an affinity towards different sequences of amino acids and a simple screen was used to determine which would be most effective for the clinical assay. A combination of Ab 1 and Ab 2 were determined to be most appropriate (see Figure 2).

Figure 2: Antibody Efficiency Screen



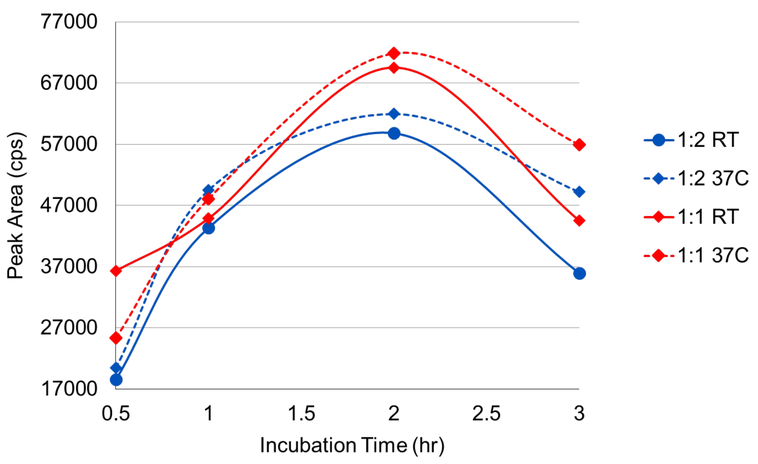
4 OPTIMISING AFFINITY

In order to optimise the volume of bead suspension:matrix an experiment was carried out by fixing the matrix volume at 500 µL and varying the volume of bead suspension. The optimal ratio of bead suspension:matrix was discovered to be 1:1, although due to the cost of the antibodies a ratio of 1:2 carries sufficient value.

The matrix volume was then optimised for the sensitivity requirements by fixing the ratio of bead suspension:matrix (1:2) but varying total volume used. A linear increase in MS response was observed, indicating that unlike the chemical SPE method a large matrix effect was not introduced by increasing the matrix volume.

In order to maximise capture the incubation time and temperature were optimised using a fixed volume of matrix and bead suspension. These parameters optimised at 2 hours incubation and at 37˚C for both 1:1 and 1:2 ratios of bead suspension:matrix (see Figure 3).

Figure 3 : Affinity Optimisation



5 EXTRACTION PROCEDURE

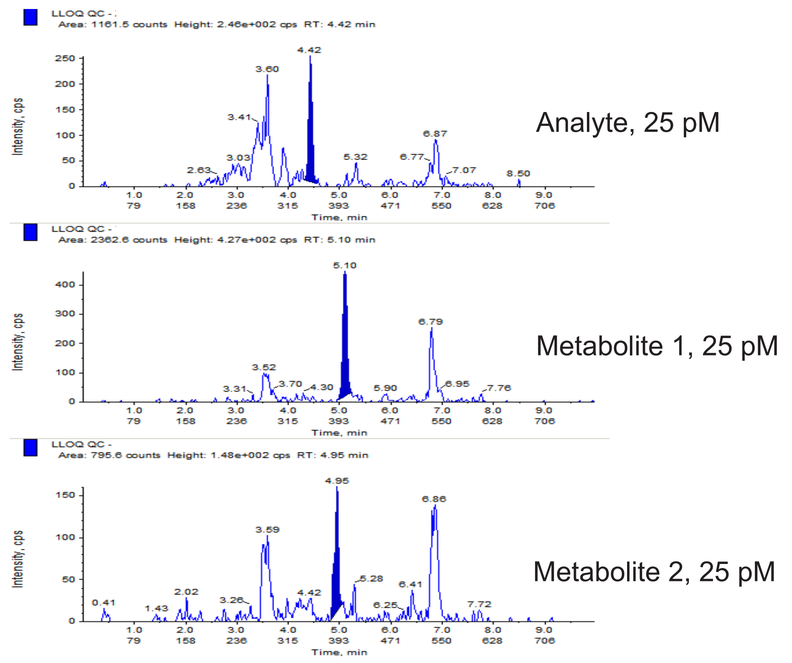
Table 2: Clinical Extraction Summary

Optimised Affinity Sample Preparation	
Matrix volume	400 µL
Bead Suspension	200 µL of functionalised Dynabeads™ suspended in phosphate buffer
Incubation	2 hours, 37°C magnetic collection
Wash	500 µL of phosphate buffer
Desorption	100 µL MeCN/H_2O/TFA, (40/60/0.25, v/v/v)
Analyses	UPLC - API6500 MRM

6 RESULTS & DISCUSSION

After commencing the Clinical study it became apparent that samples from some patients gave poor and unrecoverable chromatography. This was resolved with the use of perfusion chromatography, a form of size exclusion chromatography (see Figure 4).

Figure 4: Example Extracted Chromatography



As the problem was resolved by changing the LC retention mechanism from a reverse phase mechanism to size exclusion it was surmised that the molecular size of co-extracted material would be the root cause of the poor reverse phase behaviour. This was investigated by conducting a protein BLAST® database search. The BLAST® search returned a number of native proteins that contained the same sequence as the peptide epitopes confirming that the co-extraction of native proteins was the possible root cause for the poor reverse phase behaviour.

Table 3: Clinical Assay Summary

Validated Assay Parameters	
Assay Range	25 pM – 2500 pM
Accuracy and Precision	Within acceptable criteria
Selectivity	Within acceptable limits
Carryover	No carryover observed
Dilution Integrity	100x dilution
Matrix Factor	Consistent across six sources
Freeze/Thaw Stability	Three cycles at -80°C
Storage Stability	32 days at -80°C
Ambient Temperature Stability	24 hours on ice
Processed Sample Viability	129 hours at 4°C
Blood Stability	4 hours on ice

7 CONCLUSIONS

- An LC-MS/MS assay was validated using an optimised hybrid affinity approach and applied to clinical studies.
- An unusual non-specificity was observed using the affinity approach that required a size exclusion retention mechanism to resolve.
- Perfusion and reverse phase LC mechanisms have interesting complementarity to be explored for routine 2D separation in the BioMS space.